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BIOCHEMICAL LABORATORY METHODS

FOR

STUDENTS OF THE BIOLOGICAL SCIENCES

BY

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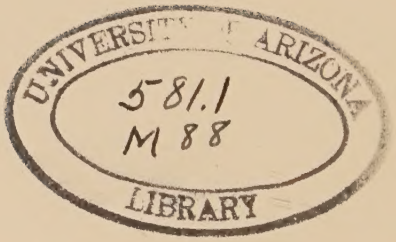

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TO

DR. ROSCOE W. THATCHER

WHO AS TEACHER AND FRIEND,

FIRST OPENED TO THE AUTHOR

THE FIELD OF PHYTOCHEMISTRY

72552

PREFACE

It is being recognized by students of the Biological Sciences that a thorough understanding of Plant Chemistry is essential to the solution of their problems. The present-day physiologist, botanist, pathologist, horticulturalist, agronomist, geneticist, bacteriologist, forester, and soil investigator finds that not only a knowledge of Biochemistry, but also practical biochemical laboratory experience is an indispensable part of his equipment. To meet this need, the University of Minnesota has for several years offered a lecture course in Phytochemistry and an accompanying laboratory course in Biochemical Laboratory Methods. The courses were planned originally as a basis upon which the senior and graduate students, who are majoring in the Division of Agricultural Biochemistry, might build a graduate program, but the needs of those students whose life work will lie in other branches of the biological sciences have been kept in view at all times.

This manual is the outgrowth of nine years' experience in Biochemical Laboratory Methods. During this time, the experiments, in mimeographed form, have been tested by regular classes. It has been assumed that the students have had previous training in both quantitative analysis and organic chemistry; however, so many have come from the Plant Science Group who have not been specifically trained as chemists, that it has seemed wise to give the experimental methods in great detail. The selection of the experiments offered has been based upon the idea of giving the student fundamental chemical principles as they relate to living processes. The materials which have been suggested for use in the experiments are chosen largely from plant sources, because the majority of the students have been primarily interested in this field. The general principles which the experiments illustrate, however, apply equally well to biological phenomena in general. In short, the course has been designed for training in research methods.

The original citation from which each experiment is taken or expanded, as well as other articles pertinent to it, are listed at the end

of the experiment, in order that the investigator may have at his disposal the fundamental references. It is hoped also that students may thus be induced to become familiar with the journal literature on the subject, and stimulated towards acquiring a good scientific attitude. By this method of appending a list of references, the author has attempted to make each experiment complete in itself. In every instance the literature citations have been carefully checked by consulting the original papers.

In arranging the material, the chapter on The Colloidal State is placed first, because a knowledge of its fundamental principles is necessary to an understanding of the chemistry of cell protoplasm, as well as the topics discussed later in the book. The chapters on Physical Chemical Constants of Plant Saps and Proteins follow, because they so characteristically illustrate many of the colloidal phenomena. Carbohydrates, Glucosides, Fats and Allied Substances, Enzymes, and Plant Pigments follow in the order named. The arrangement of topics is not intended to indicate their relative importance but is used because it makes for a simpler teaching method.

CLARENCE AUSTIN MORROW.

The foregoing paragraphs were written by the author some time before his death.

The final compilation and the completion of the manual have been made possible through the generous assistance of Mr. Truman A. Pascoe and Dr. Ross Aiken Gortner, of the Division of Agricultural Biochemistry, of the University of Minnesota, and to them I wish to express my great appreciation.

The author felt himself greatly indebted to his associates in the Division, in particular to Dr. R. A. Gortner, for his helpful suggestions, advice, and criticism throughout the development of the manual; to Dr. Paul F. Sharp for aid in the preliminary part of the work; and to Mr. Robert D. Evans, Mr. Walton B. Sinclair, and Mr. Truman A. Pascoe for their technical assistance in the experimental work. He desired to make grateful acknowledgment also, to Dr. N. C. Pervier for preparing the drawings for a number of the illustrations, and to Miss L. Mae Centerwall for her untiring efforts in assisting with the library work.

Because many important references and suggestive experiments may have been omitted from this book, the staff of the Division of Agricultural Biochemistry will greatly appreciate any criticisms or suggestions from readers.

MRS. CLARENCE A. MORROW.

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BIOCHEMICAL LABORATORY METHODS

CHAPTER I

THE COLLOIDAL STATE

I. Dialysis, Diffusion, and Osmosis

Expt. 1. Preparation of a Collodion Bag.—Thoroughly clean a 250–300 cc. Erlenmeyer flask with chromic acid cleaning solution; rinse it with 95 per cent ethyl alcohol, and allow to dry. Place in the dry flask 15–20 cc. of U. S. P. collodion, and rotate it to secure a uniform distribution of the collodion on its walls; then gradually pour the excess back into the collodion bottle. Continue rotating the flask for a few minutes until the membrane takes on a wrinkled appearance. Then carefully fill the flask with water and, after allowing it to stand for half a minute, pour it out. Cautiously loosen the collodion membrane around the mouth of the flask with the tip of a knife blade, and pour 10–25 cc. of distilled water between the collodion film and the walls of the flask. Again rotate the flask gently so that the water separates all of the collodion film from the glass; raise the collodion bag slightly; suck out the air until it collapses; and then draw it out of the flask. Test the bag for holes by filling it with water. If it leaks, make a new bag. The degree of its permeability depends partly upon the thickness of the wall, partly upon how long it is dried before water is added; for the longer the time of drying, the less permeable the membrane. The ether and alcohol in the collodion are replaced by water and there results a membrane which can be considered a hydrogel of cellulose nitrate. After a little practice, bags of two- or three-liters capacity can be prepared. Extra collodion bags can be kept under water for a long time if the water is saturated with some preservative such as toluene and the vessel covered.

Bigelow, S. L., and Gemberling, A. Collodion membranes. *J. Am. Chem. Soc.*,[1] 29, 1576–1589 (1907). The article contains a very complete bibliography.

[1] The abbreviations, used for the journals in the literature cited, are those recommended by the American Chemical Society. See *Chemical Abstracts*, 16, No. 20. I–LX (1922).

Schoep, A. Ueber ein neues Ultrafilter. *Kolloid-Z.*, 8, 80–87 (1911).

*[2] Clark, E. D. A study of Lintner soluble starch. *Biochem. Bull.*, 1, 194–206. Cf. 198 (1911). The footnote contains a description of the method for preparing collodion bags, taken from directions by Dr. Gies.

Walpole, G. S. Notes on collodion membranes for ultrafiltration and pressure dialysis. *Biochem. J.*, 9, 284–297 (1915).

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Eggerth, A. H. The preparation and standardization of collodion membranes. *J. Biol. Chem.*, 48, 203–221 (1921). A good article for a general understanding of the subject.

Looney, J. M. The preparation of flexible collodion membranes. *J. Biol. Chem.*, 50, 1–4 (1922). The addition of ethyl acetate to the collodion solution gives flexibility to the dried collodion membranes.

Nelson, J. M., and Morgan, D. P., Jr. Collodion membranes of high permeability. *J. Biol. Chem.*, 58, 305–319 (1923).

Hanke, M. T., and Koessler, K. K. A continuous dialysis or extraction apparatus which operates at reduced pressure with a constant volume of liquid. *J. Biol. Chem.*, 66, 495–499 (1925).

Expt. 2. Dialysis of Banana Extract.—Peel and grind at once 300–400 gm. of thoroughly ripe bananas (*Musa sapientum*) in a food chopper, fitted with the finest cutter. Then, using a spoon, mash the fruit in a large porcelain mortar with 5 cc. of toluene as a preservative, and with distilled water in the proportion of 100 cc. for each 400 gm. of pulp. Pour the mixture on a large fluted filter paper on a ribbed funnel and collect the filtrate in an Erlenmeyer flask containing a layer of toluene. Allow filtration to proceed from 18 to 24 hours. The extract obtained has a brownish tinge. Divide this into two equal parts and place each in a collodion bag (Expt. 1); the volume of the banana extract should always be less than one-fourth the capacity of the bag to allow for increase in volume during dialysis. Insert a rubber stopper, carrying a glass tube about 1 cm. in diameter and 30 cm. long, in the neck of each collodion bag and tie in place. Suspend one bag in tap water and the other in distilled water. Dialysis may be accomplished by a con-

[2] The asterisk (*) is used to indicate the original reference or references from which each experiment is obtained or expanded.

tinuous circulation method,[3] Fig. 1, whereby the water, whether tap or distilled, is kept constantly saturated with toluene. Partially fill a cylinder or tall bottle, A, with water and place on top a layer of toluene; then allow the water to drip in at the desired rate. It flows into the container, in which the collodion bag is suspended and which contains also a constant level siphon. Dialyze both bags for 2 or 3 days, but observe at intervals during the first 48 hours and record the results. The osmotic pressure of substances in true solution causes a flow of water into the bag during the first few hours. These substances should later dialyze out and the liquid within the bag should drop to a level governed by the osmotic pressure of the colloids.

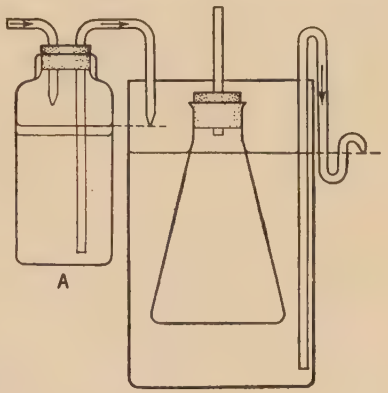

FIG. 1.

Note that practically all the banana extract, which was dialyzed against distilled water, remained in solution; while the extract dialyzed against tap water formed a gel, because of the presence of calcium salts in the tap water. These results indicate that in freeing a suspensoid sol from electrolytes, the dialysis may be carried out in tap water for the first few hours, until the greater part of the electrolytes have been removed, but thereafter, finished in distilled water. What is the distinction between dialyzing membranes and semipermeable membranes?

*GRAHAM, T. Liquid diffusion applied to analysis. *Phil. Trans. Roy. Soc., London*, 151, 183–224 (1862). Read June 13, 1861; also *J. Chem. Soc.*, 15, 216–270 (1862).

GRAHAM, T. Anwendung der Diffusion der Flüssigkeiten zur Analyse. *Ann. Chem. Pharm.*, 121, 1–77 (1862).

LOEB, J. The influence of electrolytes on the electrification and the rate of diffusion of water through collodion membranes. *J. Gen. Physiol.*, 1, 717–745 (1918–19).

LOEB, J. Influence of the concentration of electrolytes on the electrification and the rate of diffusion of water through collodion membranes. *J. Gen. Physiol.*, 2, 173–200 (1919–20).

*MCGUIRE, GRACE, and FALK, K. G. Banana gel. *J. Gen. Physiol.*, 4, 437–445 (1922).

BENCOWITZ, I., and HOTCHKISS, H. T., JR. A constant-level device for a thermostatic bath. *Ind. Eng. Chem.*, 16, 1193 (1924).

[3] Observed in the laboratory of Dr. The Svedberg at the University of Wisconsin during the Colloid Symposium, June, 1923.

Expt. 3. Dialysis of Egg Albumin in a Hardened Collodion Bag.—Prepare two collodion bags (Expt. 1) and immerse one in distilled water. *Harden* the other as follows: Dry in the air for 10 minutes; then make three more applications of the collodion, allowing each coat to dry for 10 minutes, except the last, which should dry for half an hour; fill the bag with a freshly prepared 1 per cent gelatin sol,[4] and immerse it for 15 minutes in the same sol contained in a beaker. Remove the bag from the beaker and carefully pour out its contents, draining as completely as possible. Repeat the process, substituting a freshly prepared 2 per cent formaldehyde solution for the gelatin sol. Rinse the bag carefully, both inside and out, with distilled water, and it is ready for use. Place in each collodion bag 100 cc. of egg albumin solution, or of milk serum, and dialyze in a beaker of distilled water for 48 hours. Saturate the solution used and the distilled water with toluene.

Test the dialysate from each bag for the presence of albumin by the method of Sorensen. Add 10 cc. of N acetic acid and 10 cc. of N sodium acetate solution to 100 cc. of the dialysate and heat on a water bath for half an hour. A precipitate results if the test is positive. Such small amounts of egg albumin as correspond to 0.1–0.2 mg. of protein nitrogen produce a slight opalescence. What does this experiment indicate as to the permeability of the two bags?

Egg albumin solution.—Mix thoroughly the white of a fresh egg with 250 cc. of distilled water and filter.

Milk serum.—To 300 cc. of skim milk, add an excess of rennin solution and allow it to stand for several hours in an incubator at 40° C. Filter off the casein and use the resulting serum.

*Sorensen, S. P. L., and Höyrup, Margrethe. Studies on proteins. I. On the preparation of egg-albumin solutions of well-defined compositions, and on the analytical methods used. *Compt. rend. trav. lab. Carlsberg*, 12, 12–67. Cf. 28–32 (1916).

Expt. 4. A Liquid Semipermeable Membrane.—Shake 50 cc. of a quarter saturated solution of calcium nitrate with 3 gm. of phenol until the nitrate solution becomes saturated with the latter. Pour the mix-

[4] The *percentage concentration* of the solutions used in this book is indicated by the following examples: For salts or solid substances, a 10 per cent solution means that 100 gm. of the solution contains 90 gm. of distilled water and 10 gm. of the solid substance, regardless of whether it is anhydrous or contains water of crystallization. A 10 per cent hydrochloric acid solution means that 100 gm. of solution contains 90 gm. of water and 10 gm. of hydrogen chloride. The concentration of alcohol is expressed by volume; that is, 60 per cent ethyl alcohol is prepared by diluting 60 cc. of 95 per cent ethyl alcohol to 95 cc. with distilled water.

ture into a 100 cc. graduated cylinder. The amount of phenol used is enough not only to saturate, but also to form a layer on the surface of the calcium nitrate solution a few millimeters in thickness. Next saturate 50 cc. of distilled water with phenol and cautiously introduce it above the phenol layer. Stopper the cylinder. Mark the level of the bottom of the phenol on the outside of the cylinder, and allow the system to stand. Observe from day to day. The water on both sides of the layer of phenol is soluble in it; consequently by dissolving in and passing through from either side, it can traverse the phenol layer. The calcium nitrate, however, being practically insoluble in the phenol can not pass through the phenol layer. The phenol, therefore, constitutes a perfect semipermeable membrane. As a result, the water from above passes through the phenol and in time the latter rests at the top of the liquid. A saturated solution of ammonium sulfate may be used in place of the calcium nitrate, but the experiment proceeds much more slowly.

Nernst, W. Ein osmotischer Versuch. *Z. physik. Chem.*, 6, 37–40 (1890).
*Brown, Crum. On Nernst's "osmotic experiment" and a definition of osmotic pressure. *Proc. Roy. Soc. Edinburgh*, 22, 439–440 (1897–99).

Expt. 5. Preparation of Artificial Cells.—A protein membrane suitable for permeability studies may be made by shaking a liquid, such as chloroform, which is readily volatile and slightly soluble in water, with an egg albumin solution. An exceedingly fine membrane forms at the surface of the chloroform. To prepare artificial chloroform cells, shake 3 cc. of chloroform with 10 cc. of fresh egg albumin solution (Expt. 3) in a test tube. The diameter of the droplets may be varied according to the degree of shaking. Pour these cells into a Syracuse watch glass and, if necessary, add sufficient distilled water to cover them. Examine them under the microscope from time to time, during half an hour. Try pressing a cell with a needle. Is the membrane apparent? Explain why the cell membranes become wrinkled.

To prepare chloroform-lecithin cells, shake 3 cc. of a chloroform-lecithin solution with 10 cc. of fresh egg albumin solution. Wash these artificial cells with distilled water several times and pour into a Syracuse watch glass containing sufficient water to cover them. Press a cell with a needle and observe its membranous covering and the consistency of its contents. Examine under the microscope from time to time, during the course of an hour or two, and draw the different stages observed. When the chloroform has been completely replaced by water, remove a group of the cells to another watch glass, and add several drops of

neutral red. What takes place? Why was lecithin chosen for the artificial cells?

Chloroform-lecithin solution.—Prepare a 0.0125 *M* solution of lecithin (Expt. 190) in chloroform.

Neutral red solution.—Dissolve 0.01 gm. of neutral red (dimethyl-diamino-toluphenazine) in 50 cc. of distilled water.

Harvey, E. N. A simple method of making artificial cells resembling sea urchin eggs in certain of their physical properties. *Science*, 36, 564–565 (1912).

*Harvey, E. N. A new type of artificial cell substance for permeability and other biochemical studies. *Biochem. Bull.*, 2, 50–52 (1912).

Rosett, J. Observations on a new type of artificial osmotic cell. *Plant World*, 20, 37–57 (1917).

Expt. 6. A Plant Membrane Osmometer.—The testa of either a lima bean (*Phaseolus lunatus*) or of a tepary bean makes an admirable osmotic membrane. In preparing lima beans for use, it is necessary to place several in a moist chamber for two days, while it is sufficient to soak tepary beans for a few hours. In either case select beans whose seed coats remain intact. Carefully split the testa of the lima bean through the microphyll and hilum and remove the two halves. Dry carefully between filter papers and expose to the air a few minutes; this will toughen the membrane. Each half may serve as an osmotic membrane or the whole testa of the tepary bean may be used. For the experiment use a pipet and cut off each tube just beyond the bulb. Using a rubber band fasten the membrane tightly over one end of the pipet. Fill the bulb with a 65 per cent sucrose solution, avoiding bubbles. Attach a long glass tube of 1–2 mm. diameter to the other end of the pipet, and place the bulb in a vessel of distilled water, so that the surfaces of the sugar solution and water are at the same level. Why is this necessary? Allow the experiment to proceed several days and observe occasionally. What is osmotic pressure? What application does it have in the study of plant chemistry?

Schull, C. A. Semipermeability of seed coats. *Bot. Gaz.*, 56, 169–199. Cf. 183 (1913).

Freeman, G. H. Southwestern beans and teparies. *Ariz. Agr. Expt. Sta., Bull.*, 68, 1912.

*Wilson, O. T. A plant membrane for demonstrating osmosis. *Science*, 45, 388–389 (1917).

Kremers, E. Experimental osmosis with a living membrane. *Science*, 48, 599–600 (1918). The use of a node and an internode of a dahlia stem is described.

Deuel, H. J. The digestibility of tepary beans. *J. Agr. Research*, 29, 205–208 (1924).

Expt. 7. Colloidal Flower Garden.—This experiment is based on Traube's artificial osmotic membranes, of which he originally prepared a number. The membranes, made by dropping a potassium ferrocyanide solution into a copper acetate solution, or the latter solution into a potassium silicate solution, have proved most important. Dilute a concentrated solution of sodium silicate with an equal volume of distilled water and place it in a tall beaker. Drop into this a small lump or crystal of each of certain very soluble salts, such as ferric chloride, cobaltous chloride, cupric chloride, nickelous nitrate, and manganous sulfate. The salts dissolve, forming thin layers of very concentrated solution about each lump. At the surface of each of these layers a film of insoluble silicate of the metal forms, separating it from the sodium silicate. This film serves as an osmotic membrane. The cell formed about each lump behaves like a growing seed, for it appears immediately to sprout and send up shoots toward the surface of the sodium silicate, producing what may be called a colloidal flower garden. Explain this phenomenon.

*Traube, M. Experimente zur Theorie der Zellenbildung und Endosmose. *Arch. Anat. Physiol. wiss. Med.*, S. 87–165. Cf. 124 (1867).

*Talbot, H. P., and Blanchard, A. A. The electrolytic dissociation theory with some of its applications. p. 8. The Macmillan Company, New York, 1907.

II. Optical Properties

Expt. 8. Tyndall Effect.—One of the most effective methods for studying the properties of suspensions, suspensoid sols, smokes, and mists is by means of a beam of light. In 1856 Faraday observed that transparent gold sols showed turbidity and luminosity when a powerful beam of light was projected through the liquid. Tyndall subsequently applied this method to the study of smokes and fumes in the atmosphere, so that the phenomenon is usually called by his name, the Tyndall effect or the Tyndall cone. In the study of the Tyndall effect, it is observed that the larger the colloidal particles, the brighter they appear. Also the more intense the light provided, the larger the number of small particles which will be visible. Because there is a difference in the index of refraction between the disperse phase and the dispersion medium, suspensoid sols always show the Tyndall effect, while emulsoid sols as a rule do not show it.

A simple apparatus for use in a dark room can be prepared by placing a 100–200 watt electric light in a box with a hole about 5 mm. in diameter opposite the center of the bulb. If a projection lantern is avail-

able, it should be used because it furnishes a much more intense light.

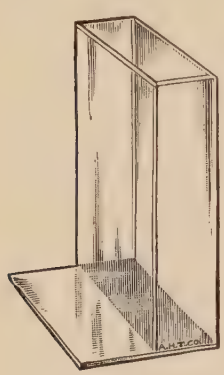

Fig. 2.

Pour the suspensoid sol to be examined into an absorption cell, such as is illustrated in Fig. 2. The cell, $10 \times 10 \times 3$ cm., is of polished plate glass. Set it in the path of the beam of light. Make the observations at right angles to the incident beam. The Tyndall effect is observed in the suspensoid sol when thus illumined from one side. Each student should examine two of the suspensoid sols, which are subsequently prepared, for the Tyndall effect. Also observe the distilled water used in their preparation. What is *optically clear* water? How may such water be obtained?

*Faraday, M. Experimental relations of gold (and other metals) to light. *Phil. Trans. Roy. Soc., London*, 147, 145–181. Cf. 160 (1858). Received Nov. 15, 1856.

Tyndall, J. On the blue colour of the sky, the polarization of skylight, and on the polarization of light by cloudy matter generally. *Proc. Roy. Soc. (London)*, 17, 223–233 (1868–69); or *Phil. Mag.* [4], 37, 384–394 (1869).

Tolman, R. C., and Vliet, E. B. A Tyndallmeter for the examination of disperse systems. *J. Am. Chem. Soc.*, 41, 297–300 (1919).

Tolman, R. C., Reyerson, L. H., Vliet, E. B., Gerke, R. H., and Brooks, A. P. Relation between the intensity of Tyndall beam and concentration of suspensions and smokes. *J. Am. Chem. Soc.*, 41, 300–303 (1919).

Tolman, R. C., Vliet, E. B., Peirce, W. McG., and Dougherty, R. H. The disappearance of smoke in a confined space. *J. Am. Chem. Soc.*, 41, 304–312 (1919).

Martin, W. H. The scattering of light by dust-free liquids. *J. Phys. Chem.*, 24, 478–492 (1920).

Burton, E. F. The physical properties of colloidal solutions. 2nd ed., pp. 96–121. Longmans, Green and Company, London, 1921.

Expt. 9. The Ultramicroscope.—The ultramicroscope is an apparatus provided with an intense dark-ground illumination for the observation of particles too small to be seen by transmitted light.

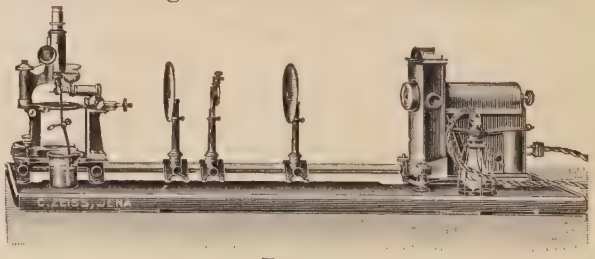

Fig. 3.

(*a*) In 1903 Siedentopf and Zsigmondy devised an instrument, which they called the *slit ultramicroscope*. It has since been somewhat modified and the later form is illustrated in Fig. 3. The apparatus is mounted upon a base board, fitted with an optical

bench, one meter long. The light rays from an arc lamp emerge through a diaphragm, parallel to the optical bench. The projection lens, adjacent to the arc lamp, is mounted in a round metal screen to shut off the light coming from the sides, and is placed on the bench so that the front of the lens faces the microscope. Next to it is the adjustable micrometer slit which is moved back and forth on the optical bench until the projection lens throws into it a good image of the source of light. Beyond the adjustable micrometer slit is another projection lens, which is also provided with a metal screen. The preparation to be examined is placed in a small observation cell, Fig. 4, which is attached to the objective of the observation microscope by means of a special holder, so that it can be raised or lowered by adjusting screws. The cell is rectangular in cross-section and has a thistle funnel, g, fused

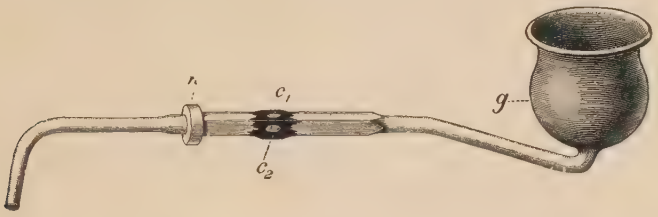

FIG. 4.

on at one end. To the other end is attached a piece of rubber tubing, provided with a clamp to prevent the escape of the solution. The quartz window, c_2, should face the source of light, and c_1, the lens of the objective. Using this cell, any number of colloidal solutions may be examined without further adjustment of the apparatus. However, the cell should be washed thoroughly with distilled water or chromic acid cleaning solution and distilled water before being filled with a new sol. Care must be exercised in filling it to make sure that there are no air bubbles in front of the windows. Place a drop of distilled water, the immersion medium, on the upper window before inserting the cell in its position; then carefully wipe off the quartz window, c_2, for the least trace of water will spoil the illumination. Until the immersion water and the colloidal solution in the cell have attained the same temperature, there will be a streaming in one direction across the field. For a more detailed description with directions for the use of the Siedentopf and Zsigmondy ultramicroscope and its attachments, see pamphlet, Mikro 229, Carl Zeiss,[5] Jena, Germany.

[5] A representative of this company is located at 110 East 23rd Street, New York City.

Using this ultramicroscope, observe the gold sol (Expt. 10). The particles will be seen as bright discs with vigorous Brownian movement. What is the Brownian movement? Explain its cause. What are the methods used for measuring the size of ultramicroscopic particles?

Brown, Robert. A brief account of microscopical observations made in the months of June, July, and August, 1827, on the particles contained in the pollen of plants; and on the general existence of active molecules in organic and inorganic bodies. *Phil. Mag.*, 161–173 (1828).

Brown, Robert. Additional remarks on active molecules. *Phil. Mag.*, 6, 161–166 (1829).

* Siedentopf, H., und Zsigmondy, R. Über Sichtbarmachung und Grössenbestimmung ultramikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser. *Ann. Physik* [4], 10, 1–39 (1903).

Perrin, J. Brownian movement and molecular reality. Translated by F. Soddy from *Ann. chim. phys.* [8], Sept., 1909. Taylor and Francis, London, 1910.

Jentzsch, F. Der Ultrakondensor. Ein neuer Apparat für ultramikroskopische Untersuchungen. *Ber. physik. Ges.*, 8, 992–994 (1910); also *Physik. Z.*, 11, 1001–1002 (1910).

Zsigmondy, R., und Bachmann, W. Handhabung des Immersionsultramikroskops. *Kolloid-Z.*, 14, 281–295 (1914).

Zsigmondy, R. Colloids and the ultramicroscope. A manual of colloid chemistry and ultramicroscopy. Translated by J. Alexander. John Wiley & Sons, New York, 1914.

Bancroft, W. D. Brownian movements. *J. Phys. Chem.*, 22, 273–299 (1918).

Wells, P. V., and Gerke, R. H. An oscillation method for measuring the size of ultramiscroscopic particles. *J. Am. Chem. Soc.*, 41, 312–329 (1919).

King, G. Ultra-microscopy: degree of dispersion-measurement with the ultramicroscope. Third report on colloid chemistry and its general and industrial applications. *Brit. Assoc. Advancement Sci., Reports*, pp. 31–40 (1920).

Ostwald, W. Rechentafel zum Stokes'schen Gesetz. *Kolloid-Z.*, 26, 213–215 and 217 (1920).

Burton, E. F. The physical properties of colloidal solutions. 2nd ed., pp. 28–95; 122–131. Longmans, Green and Company, London, 1921.

Berek, M. Zur Theorie der Spiegelkondensoren für Dunkelfeldbeleuchtung und Ultramikroskopie. Vervollkommungen bisheriger Einrichtungen. *Z. wiss. Mikrosk.*, 40, 225–240 (1923).

Jentzsch-Graefe, F. Über Dunkelfeld- und Ultramikroskopie. Kraus und Uhlenhuth: Handbuch der mikrobiologischen Technik. Bd. 1. S. 49–69. Urban und Schwarzenberg, Berlin, 1923.

Newell, L. C. Robert Brown and the discovery of the Brownian movement. *Ind. Eng. Chem.*, 15, 1279–1281 (1923).

(*b*) The slit ultramicroscope is most convenient for the examination of ultramicroscopic particles, since several colloidal sols may be observed in rapid succession; but the *cardioid ultramicroscope* of Siedentopf is satisfactory with most suspensoid sols.

Use a microscope which has a condenser, but replace this with a cardioid condenser. Provide an arc lamp as the source of light and

regulate the illuminating lens so that the emerging pencil of light falls upon the mirror of the microscope. From it the light passes vertically upward into the cardioid condenser. This has a *nearly aplanatic dark ground* and may be defined as a bispherical reflecting condenser. In a strict mathematical sense, its rays are not brought into a true aplanatic focus; but the theoretical defect is too slight to be of any practical consequence. Figure 5 is a diagrammatic representation of the direction of the rays in a cardioid condenser, beneath a photomicrograph, which shows the intersecting rays transmitted through a plate of uranium glass. Observe that the rays first undergo divergence at the inner convex spherical surface, after which they are reflected at the outer concave spherical surface and are brought to focus within the uranium glass. There should be no air between the condenser and the cell containing the colloidal sol to be examined, so a film of an *immersion liquid*, such as cedar oil, free from air bubbles, should be applied. For a more detailed description of the Siedentopf cardioid ultramicroscope, see pamphlet, Mikro 306, Carl Zeiss, Jena.

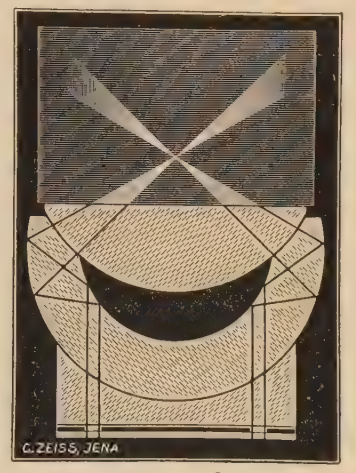

FIG. 5.

*SIEDENTOPF, H. Über einen neuen Fortschritt in der Ultramikroskopie. *Ber. physik. Ges.*, 8, 6–47 (1910).

*SIEDENTOPF, H. Über bisphärische Spiegelkondensoren für Ultramikroskopie. *Ann. Physik* [4], 39, 1175–1184 (1912).

III. SUSPENSOID SOLS

Success in the preparation of suspensoid sols depends upon the cleanliness of the vessels used and the degree of purity of the distilled water. The vessels should be of hard glass, such as Pyrex or Jena, cleaned with chromic acid cleaning solution, rinsed with distilled water, and finally either rinsed again with hot specially distilled water or steamed for several hours. The solution should also be stirred with hard glass stirring rods. The *specially distilled water* should be freshly prepared, and condensed in quartz, block tin, or hard glass tubes. The supply of water in the condenser jacket should be cut down so that the volatile substances present will not be condensed and the water will issue steaming.

A. CONDENSATION METHODS

Reduction

Expt. 10. Gold Sol by Formaldehyde.—Place 120 cc. of specially distilled water in a 300 cc. Pyrex beaker and bring it to boiling. While heating, add 2.5 cc. of gold chloride solution and 3–3.5 cc. of *freshly prepared* 0.18 *N* potassium carbonate solution and stir. As soon as the boiling point is reached, remove the flame. Then add 3–5 cc. of formaldehyde solution, a drop at a time, allowing a few seconds to intervene and stirring between each addition. The reaction begins in a few seconds. Continue the addition of the formaldehyde until a deep red color results. Note carefully and explain the series of color changes which occur during the reduction. Observe the sol by both transmitted and reflected light. Observe also for the Tyndall effect (Expt. 8). What electric charge does the sol carry? Why does its preparation in a slightly alkaline solution make it more *stable*? The gold sol used for the determination of the gold number (Expt. 49) is prepared by this method.

Gold chloride solution.—Dissolve 3.43 gm. of pure gold in *aqua regia* in a casserole and evaporate to dryness on a steam bath. Add a small quantity of concentrated hydrochloric acid, and again evaporate to dryness; then add distilled water and evaporate to dryness a third time. Finally dissolve the resulting chlorauric acid, $HAuCl_4$, $3H_2O$, in sufficient specially distilled water to make one liter. This gives a concentration of 6 gm. per liter.

Formaldehyde solution.—Dilute 0.6 cc. of 37–40 per cent formaldehyde with 200 cc. of specially distilled water. The solution should be freshly prepared.

Zsigmondy, R. Ueber wässerige Lösungen metallischen Goldes. *Ann.*, 301, 29–54 (1898).

*Zsigmondy, R. Die hochrothe Goldlösung als Reagens auf Colloide. *Z. anal. Chem.*, 40, 697–719 (1901).

*Schulz, F. N., und Zsigmondy, R. Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweissstoffen. *Beitr. Chem. Physiol. Pathol.*, 3, 137–160. Cf. 141 (1903).

Ostwald, W. Ueber Farbe und Dispersitätsgrad kolloider Lösungen. *Kolloid-chem. Beihefte*, 2, 409–488 (1910–11). The relationship between color and degree of dispersion is discussed.

Expt. 11. Gold Sol by Phosphorus.—To prepare a gold sol by phosphorus Zsigmondy combines two methods, his own formaldehyde method (Expt. 10) and that of Faraday, who used a solution of white phosphorus in ether. By this combination, the sol obtained has a high

degree of dispersion and is very sensitive to electrolytes. Place 120 cc. of specially distilled water in a 300 cc. Pyrex beaker; add 2.5 cc. of gold chloride solution and 3–3.5 cc. of freshly prepared 0.18 *N* potassium carbonate solution (Expt. 10). Next add, drop by drop, 0.5 cc. of a saturated solution of white phosphorus in ether. Reduction takes place at room temperature but the reaction is slow; the liquid first becomes brownish-red and then gradually changes to a bright red, without the slightest turbidity either in transmitted or reflected light. A saturated solution of phosphorus in carbon tetrachloride may be used in place of the phosphorus in ether.

*Faraday, M. Experimental relations of gold (and other metals) to light. *Phil. Trans. Roy. Soc., London*, 147, 145–181. Cf. 159–160 (1858). Received Nov. 15, 1856.

Svedberg, T. Die Methoden zur Herstellung kolloider Lösungen anorganischen Stoffe. S. 65–66. Theodor Steinkopff, Dresden, 1909.

*Zsigmondy, R. Colloids and the ultramicroscope. Translated by J. Alexander. 1st ed., pp. 126–128. John Wiley & Sons, New York, 1914.

Expt. 12. Gold Sol by Phenylhydrazine.—Place 300 cc. of specially distilled water in a 500 cc. Pyrex beaker and add 1.25 cc. of gold chloride solution (Expt. 10). Then add 0.2–0.5 cc. of freshly prepared phenylhydrazine hydrochloride solution, a drop at a time, from a pipet, allowing a few seconds to intervene and stirring between each addition. Note the changes in color. Continue to add the reducing solution, drop by drop, and observe the color changes when 5 cc. have been added and again after the addition of 10–12 cc. The solution should now be a deep blue color. Explain this series of color changes. Allow the solution to stand for 48 hours or longer, and observe the precipitate which separates. What is this?

Phenylhydrazine hydrochloride.—The phenylhydrazine hydrochloride should be perfectly white. This salt rapidly decomposes and darkens unless it is very pure and dry. It is prepared from phenylhydrazine [6] which has been freshly purified by distillation under diminished pressure (p. 136). Dissolve the phenylhydrazine in twelve volumes of 95 per cent ethyl alcohol and precipitate as the hydrochloride by the addition of a slight excess of concentrated hydrochloric acid. Filter the hydrochloride on a Büchner funnel, sucking as dry as possible; wash thoroughly, first with a mixture of ethyl alcohol and ether, and then with ether, until the salt is snow white. Dry on a filter paper in a warm place for half an hour, and then at 100° C. for an hour. Preserve the

[6] Phenylhydrazine is poisonous. Its vapors should not be breathed; if it comes in contact with the skin, it produces an intolerable itching. Dilute acetic acid will remove it.

dry salt in a tightly stoppered amber-colored bottle. If only an impure sample of phenylhydrazine hydrochloride is available, it may be purified in the following manner: Dissolve 25 gm. of the impure salt in boiling distilled water; slightly acidify with hydrochloric acid; decolorize the solution with the vegetable decolorizing carbon, Norit, at boiling temperature, and filter at once. Allow the clear solution to stand over night in a refrigerator at 1 or 2° C., so that crystallization may take place. Filter on a Büchner funnel, and dry as described above. To prepare the solution for this experiment dissolve 1 gm. of phenylhydrazine hydrochloride in 250 cc. of distilled water.

*Gutbier, A., und Resenscheck, F. Über das flüssige Hydrosol des Goldes. II. *Z. anorg. Chem.*, 39, 112–114 (1904); or Svedberg, T. Die Methoden zur Herstellung kolloider Lösungen anorganischer Stoffe. S. 112–114. Theodor Steinkopff, Dresden, 1909.

*Mulliken, S. P. A method for the identification of pure organic compounds. Vol. I, p. 32. John Wiley & Sons, New York, 1905. The preparation of pure phenylhydrazine hydrochloride is described in the footnote.

Expt. 13. Gold Sol by Tannin.—Place 200 cc. of specially distilled water in a Pyrex beaker; add 1 cc. of gold chloride solution (Expt. 10) which has been neutralized to litmus paper by the addition of freshly prepared 0.18 *N* potassium carbonate solution, and 1 cc. of a 1 per cent tannin solution. Heat the mixture gradually to boiling, stirring constantly. A cherry red color develops as it becomes hot. If the red color does not appear immediately after heating, add more gold chloride and tannin alternately; heat and stir. Observe the sol in both transmitted and reflected light. Ordinary distilled or tap water may also be used for this experiment.

Reduction will take place in the cold if a larger proportion of tannin solution is used. To 200 cc. of water, containing the gold chloride solution, add gradually, while stirring, 6–10 cc. of tannin solution; or add powdered tannin from a spatula. Stir thoroughly. This experiment makes a good lecture demonstration.

*Ostwald, W. An introduction to theoretical and applied colloid chemistry. Translated by M. H. Fischer. 2nd ed. pp. 23–24. John Wiley & Sons, Inc., New York, 1922.

Hydrolysis

A number of different sols have been prepared by the hydrolysis of salts. All salts, theoretically, undergo hydrolysis, but this is not readily recognized unless either the acid of the salt, or the base, or both are weak electrolytes. The hydrolysis of ferric chloride was investigated by Krecke, who found that with solutions containing more than

2 per cent of ferric chloride the hydrolysis is reversed on cooling, while with less than 1 per cent it is irreversible. The temperature, the concentration of the solution, and the rate of heating are all important factors in determining both the point at which hydrolysis begins and the extent to which it is carried. A number of different ferric oxide hydrosols are, therefore, possible.

Expt. 14. Ferric Oxide Sol.—(*a*) Heat 250 cc. of 1 per cent ferric chloride solution in a beaker. Raise the temperature to 90° C. about 1° C. per minute. At what temperature does hydrolysis begin? What proportion of the solution is hydrolyzed? Place the sol in a collodion bag and dialyze (Expt. 2) it against distilled water in a beaker until the next laboratory period. Test the diffusate for both chloride and ferric ions. Complete the dialysis in running distilled water. What substances are removed by this process? Will prolonged dialysis remove all of the chloride?

(*b*) Heat 500 cc. of distilled water to vigorous boiling; add, with constant stirring, 2 cc. of a 30 per cent ferric chloride solution. The liquid turns a deep reddish-brown and remains perfectly clear. Observe the difference in the appearance of this sol and the one prepared in (*a*). The color of a ferric oxide sol is influenced by at least three factors: concentration, size of particle, and the depth of the liquid examined. Observe a portion of the sol for the Tyndall effect (Expt. 8). Dialyze until the dialysate is free from chlorides. Test a portion of the dialyzed sol for the ferric ion by adding a few drops of potassium ferrocyanide solution. The amount of hydrochloric acid formed by hydrolysis is only a small fraction of that required for coagulation. Bancroft points out that the sol owes its stability to the adsorption of the cation. If the cation is readily adsorbed while the anion is only slightly so, a high concentration of electrolyte will be necessary before the degree of adsorption of the anion becomes equal to that of the cation. This must happen before coagulation can take place. To accomplish this, add concentrated hydrochloric acid, drop by drop, to 25 cc. of the cooled sol until it becomes turbid or opaque to reflected light. Dialyze (Expt. 2) the remainder of the ferric oxide sol against distilled water until most of the chloride ions have been removed. Only experience can tell how far the dialysis may be carried without coagulation. Test a portion of the dialyzed sol for the chloride ion as follows: dissolve it in dilute nitric acid and add a few drops of silver nitrate solution. What electric charge is carried by a ferric oxide sol? What factors determine the *stability* of a sol? What is the complex theory of colloids? Preserve the dialyzed sol for use in the experiments on coagulation (Expts. 35, 36).

*Krecke, F. W. Die Dissoziationserscheinungen wässeriger Lösungen von Eisenchlorid. *J. prakt. Chem.* [2], 3, 286–307 (1871); or Svedberg, T. Die Methoden zur Herstellung kolloider Lösungen anorganischer Stoffe. S. 268–275. Theodor Steinkopff, Dresden, 1909.

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Beans, H. T., and Eastlack, H. E. The electrical synthesis of colloids. *J. Am. Chem. Soc.*, 37, 2667–2683 (1915). The article contains a discussion of the complex theory of colloids.

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Bradfield, R. A centrifugal method for preparing colloidal ferric hydroxide, aluminum hydroxide and silicic acid. *J. Am. Chem. Soc.*, 44, 965–974 (1922).

Browne, F. L. The constitution of ferric oxide hydrosol from measurements of the chlorine- and hydrogen-ion activities. *J. Am. Chem. Soc.*, 45, 297–311 (1923). This article is a discussion of the electric charge carried by a ferric oxide sol.

Precipitation

Expt. 15. Prussian Blue Sol.—To illustrate the relation between the concentration of the reacting substances and the size of particles, use three different concentrations of solutions, very dilute, medium, and concentrated. For this purpose, a freshly saturated potassium ferrocyanide solution and a 30 per cent ferric chloride solution are provided.

(*a*) Dilute three drops of each of the original potassium ferrocyanide and ferric chloride solutions to 250 cc. with distilled water; then pour the latter solution into the former, while stirring continually. Filter the resulting mixture. Explain the result.

(*b*) Dilute 10 cc. of each of the original solutions to 50 cc. with distilled water and mix quickly and thoroughly in the order indicated in (*a*). Filter the mixture and explain the results.

(*c*) Pour into a beaker quickly and at the same time, 25 cc. portions of each of the original potassium ferrocyanide and ferric chloride solutions. Mix a small quantity of the resulting substance with 200 cc. of distilled water and filter. Explain the result.

Show the results of the use of the different concentrations by plotting a curve with the concentration of the reaction mixture as abscissae and the size of the particles as ordinates. What is the application of this series of experiments to quantitative analysis? von Weimarn prepared colloidal barium sulfate by mixing solutions of manganese sulfate and barium thiocyanate of different concentrations.

von Weimarn, P. P. Zur Lehre von den Zustanden der Materie. Bd. 1: Text. S. 10–25, Bd. 2: Atlas. 100 Figs. Theodor Steinkopff, Dresden, 1914.

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Taylor, W. W. The chemistry of colloids and some technical applications. 2nd ed., pp. 170–179. Longmans, Green and Company, New York, 1921.

*Ostwald, W. An introduction to theoretical and applied colloid chemistry. Translated by M. H. Fischer. 2nd ed., pp. 25–26. John Wiley & Sons, Inc., New York, 1922.

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B. DISPERSION METHODS

Expt. 16. Electrical Dispersion. Bredig's Method.—The preparation of colloidal solutions by passing an arc between two similar electrodes under distilled water was devised by Bredig in 1898. He found that, when gold wires were used, red or violet liquids which were similar to Zsigmondy's gold sols (Expt. 10) were produced. Thus a gold, silver, or platinum sol may be prepared by passing a direct current of 40–70 volts through a variable resistance. If gold wires are submerged in distilled water, a violet or blue sol will be obtained; but if a weak alkaline solution, such as 0.001 *N* sodium hydroxide, is used a pink or red sol will result. Explain these different results. To obtain the arc, immerse the wires in the liquid, bring them into contact beneath the surface and then slowly separate them until an arc is established between them. If the wires are drawn too far apart it will be broken. This arc disintegrates the metal. In the passage of the current, the metallic gold tends to leave the cathode in very small particles and deposit on the anode; it does not all arrive, and consequently the cathode loses more in weight than the anode gains. The metal particles lost on the way remain dispersed in the liquid, forming a colloidal solution.

*Bredig, G. Darstellung colloidaler Metalllösungen durch elektrische Zerstäubung. *Z. angew. Chem.*, 951–954 (1898).

Bredig, G., und Haber, F. Ueber Zerstäubung von Metallkathoden bei der Electrolyse mit Gleichstrom. *Ber.*, 31, 2741–2752 (1898).

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Burton, E. F. Forces regulating the size of colloidal particles. Colloid symposium monograph. First national symposium on colloid chemistry, pp. 174–186. Department of Chemistry, University of Wisconsin, Madison, 1923.

Kraemer, E. O., and Svedberg, T. Formation of colloid solutions by electrical pulverization in the high-frequency alternating current arc. *J. Am. Chem. Soc.*, 46, 1980–1991 (1924).

Expt. 17. Arsenious Sulfide Sol by Peptization.—Suspend 1.5 gm. of arsenious oxide in 750 cc. of distilled water and boil until dissolved. Cool the solution nearly to room temperature and pass into it a slow steam of pure hydrogen sulfide gas until the colloidal solution, which is formed, becomes an intense, opalescent yellow. Shake the solution occasionally during the process. Observe the sol by both transmitted and reflected light and record the results. Remove the excess of hydrogen sulfide by bubbling air through the sol and preserve it for the experiments on coagulation (Expts. 35, 36). If necessary, filter the sol before use to remove traces of precipitated arsenious sulfide. What electric charge does this sol carry?

*Picton, H. The physical constitution of some sulfide solutions. *J. Chem. Soc.*, 61, 137–148 (1892).

Linder, S. E., and Picton, H. Solution and pseudo-solution. Part II. Some physical properties of arsenious sulfide and other solutions. *J. Chem. Soc.*, 67, 63–73 (1895).

Freundlich, H. Über das Ausfallen kolloidaler Lösungen durch Elektrolyte. *Z. physik. Chem.*, 44, 129–160. Cf. 132–134 (1903).

Expt. 18. Barium Carbonate Sol by Peptization.—Pass into 10 cc. of a 4 per cent solution of barium monoxide in methyl alcohol, a stream of carbon dioxide from a cylinder, until a thick gel of barium carbonate forms. Then pass in more carbon dioxide until the carbonate is peptized and a colloidal sol results. This has the consistency of thick collodion. Explain the change which occurred.

*Neuberg, C., und Neimann, E. Über gelatinöse anorganische Erdalkalisalze. *Biochem. Z.*, 1, 166–176 (1906).

Neuberg, C., und Rewald, B. Ueber kolloide und gelatinöse Verbindungen der Erdalkalien. *Kolloid-Z.*, 2, 321–324; 354–357 (1907–08). The authors suggest that the ease with which the alkaline earths form colloidal sols may be of biological importance in plant assimilation and the reduction of carbon dioxide.

Expt. 19. Aluminum Oxide Sol by Peptization.—With a pipet, measure into a 300 cc. beaker 25 cc. of aluminum chloride solution; add 100 cc. of distilled water, and precipitate with ammonium hydroxide at the boiling point, using a slight excess. Be sure that the solution is alkaline to litmus. Filter through an 11 cm. quantitative filter paper and wash with hot distilled water until free from chlorides. Wash the precipitate into a 500 cc. Erlenmeyer flask with 250 cc. of hot distilled water. Heat the contents of the flask to boiling and add from a buret small quantities of 0.05 *N* hydrochloric acid, boiling several minutes after each addition. Continue this process until the hydroxide is completely transformed into a homogeneous colloid, replacing, if necessary,

the water lost by evaporation. Observe the sol for the Tyndall effect (Expt. 8). From the volume of hydrochloric acid used, calculate the number of milligrams required for peptization. The amount needed depends largely upon the age of the aluminum hydroxide gel. How does the amount of hydrochloric acid used for the peptization of aluminum hydroxide compare with that required by their combining weights? Explain the formation of the sol according to the theory of peptization. What electric charge does it carry? In what other ways may peptization of aluminum hydroxide be brought about?

Aluminum chloride solution.—Prepare a 5 per cent solution of aluminum chloride, $AlCl_3$, $6H_2O$. Determine its concentration in terms of aluminum oxide according to the usual gravimetric method. Record on the bottle the amount of aluminum oxide contained in 25 cc. of the solution.

Müller, A. Ueber die Herstellung kolloider Lösungen durch Anätzung von Hydrogelen. *Kolloid-Z.*, 2, Sup. 1, VI–VIII (1907–08).

*Müller, A. Über die Herstellung von Metalloxydhydrosolen durch Anätzung (Peptisation) der Gele. *Z. anorg. Chem.*, 57, 311–322. Cf. 312–313 (1908).

Zsigmondy, R. The chemistry of colloids. Translated by E. B. Spear. pp. 74–81. John Wiley & Sons, Inc., New York, 1917.

Weiser, H. B. Hydrous oxides. II. *J. Phys. Chem.*, 24, 505–538 (1920).

Bradfield, R. A centrifugal method for preparing colloidal ferric hydroxide, aluminum hydroxide and silicic acid. *J. Am. Chem. Soc.*, 44, 965–974 (1922).

Expt. 20. Silver Halide Sols by Peptization.—These sols are prepared by mixing solutions of silver nitrate and a soluble halide. A slight excess of either the silver or halide ion produces an electrically charged sol, the character of which depends upon the ion adsorbed. Number a series of test tubes consecutively from one to five. Place in them 10 cc. quantities of freshly prepared potassium iodide or silver nitrate solutions as follows: in (1) and (2), 0.05 *N* potassium iodide and in (3), (4), and (5), 0.05 *N* silver nitrate. To tube (1) now add 15 cc., and to (2) 10.1–10.5 cc. of silver nitrate solution; to (3) add 10 cc. of potassium iodide, to (4) 10.1–10.5 cc., and to (5) 15 cc. of the same solution. Shake the tubes thoroughly and allow them to stand. Illustrate the results of the experiment by a diagram and explain them according to the theory of peptization. What electric charge is carried by each sol?

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*Lottermoser, A. Über kollöidale Salze I. (Silbersalze). *J. prakt. Chem.* [2], 72, 39–56 (1905).

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Lottermoser, A., und Rothe, A. Beiträge zur Kenntnis des Hydrosol und Hydrogelbildungsvorganges II. Adsorption von Silbernitrat und Jodkalium durch amorphes Jodsilber. *Z. physik. Chem.*, 62, 359–383 (1908).

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Weiser, H. B. The effect of adsorption on the physical character of precipitated barium sulfate. *J. Phys. Chem.*, 21, 314–333 (1917).

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IV. Emulsions

An emulsion may be defined as a disperse system, both phases of which are liquid at the ordinary temperature. The simplest form consists of a mixture of two immiscible liquids. Two types of emulsions, which have entirely different properties, may be produced from oil and water. One is an oil-in-water emulsion, in which the oil is dispersed as small globules in a continuous water phase; the other is a water-in-oil emulsion, in which the water is dispersed as small globules in a continuous oil phase. It has been definitely established that three phases are essential for the formation of a *stable* emulsion: first, a liquid, such as an oil; second, water; and third, an emulsifying agent. The type of emulsion formed depends upon the emulsifying agent. If it is wet by water more easily than by oil, water will be the continuous phase and oil, the dispersed phase; but if it is wet by oil more easily than by water, the reverse is true. Or, according to Bancroft, if the interfacial tension between the water and the emulsifying agent is lower than that between the oil and the emulsifying agent, the water will be the continuous phase and oil, the dispersed phase. The most common emulsifying agents are colloidal substances, such as gums, soaps, egg albumin, lecithin, and casein.

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Langmuir, I. The Constitution and Fundamental Properties of Solids and Liquids. I. Solids. *J. Am. Chem. Soc.*, 38, 2221–2295 (1916).

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Sherrick, J. L. Oil-field emulsions. *J. Ind. Eng. Chem.*, 12, 133–139 (1920). These emulsions belong to the water-in-oil type.

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Finkle, P., Draper, H. D., and Hildebrand, J. H. The theory of emulsification. *J. Am. Chem. Soc.*, 45, 2780–2788 (1923); reprinted in Colloid Symposium Monograph. I. pp. 196–209. Department of Chemistry, University of Wisconsin, 1923.

Harkins, W. D. The orientation of molecules in the surfaces of liquids. Colloid Symposium Monograph. II. pp. 141–173. Chemical Catalog Company, New York, 1925.

Expt. 21. An Oil-in-water Emulsion: an Artificial Milk.—Place 7.5 gm. of powdered U. S. P. gum acacia (gum arabic) in a large mortar, adding gradually 10–12.5 cc. of distilled water, and triturating constantly, until the acacia is thoroughly hydrated and a smooth preparation is obtained. To this add 10 gm. of refined cottonseed oil, 1 cc. at a time, with thorough grinding after each addition until all the oil is emulsified. A peculiar crackling noise is heard when the emulsion is complete. Corn oil, melted butter fat, or lard may also be used. Next add, with continued trituration, distilled water beginning with about 5 cc. and gradually increasing the dilution until a volume of 250 cc. is reached. If melted butter fat or lard is used, the distilled water must be warmed before adding. The resulting milk contains 4 per cent of fat. Cream will rise on the milk, but this can be readily re-emulsified by gentle shaking; the milk must first be warmed if butter fat or lard has been used. This artificial milk will keep for several weeks before the emulsion breaks. It is an ideal substrate for the estimation of

lipase activity (Expt. 206). The acacia is a strongly hydrophylic colloid. Why is such a large amount used? How is an emulsion stabilized? Account for the color of the emulsion.

Another method, known as the Continental, may be used for preparing pharmaceutical emulsions. For it, use definite quantities of oil, gum, and water, and make what is called the *emulsion nucleus*. This on dilution with any quantity of water forms a good emulsion. By this method, all of the emulsifying agent is hydrated at one time, and in the presence of the dispersed phase. Place 4 gm. of powdered U. S. P. gum acacia (gum arabic) in a large mortar, add 8 gm. of cottonseed oil, and triturate thoroughly until a smooth preparation is obtained. Then add 6 cc. of distilled water, all at once, and again triturate thoroughly until a thick creamy nucleus is formed. Dilute this emulsion nucleus with distilled water until a volume of 200 cc. is obtained. Preserve the emulsion prepared by either method for use in Experiment 24.

*Fischer, M. H., and Hooker, Marian O. Fats and fatty degeneration. A physico-chemical study of emulsions and the normal and abnormal distribution of fat in protoplasm. pp. 29–30; 108–111. John Wiley & Sons, Inc., New York, 1917.

*Roon, L., and Oesper, R. E. A contribution to the theory of emulsification based on pharmaceutical practice. *J. Ind. Eng. Chem.*, 9, 156–161 (1917). The Continental method is described.

*Palmer, L. S. The influence of various antiseptics on the activity of lipase. *J. Am. Chem. Soc.*, 44, 1527–1538. Cf. 1529 (1922).

Expt. 22. A Water-in-oil Emulsion.—Place 0.25 gm. of gum dammar in a large mortar, add 10 cc. of either refined cottonseed or corn oil, and triturate thoroughly until a smooth preparation is obtained. To this add 20 cc. of distilled water, 1 cc. at a time, triturating thoroughly after each addition until all of the water is emulsified. What kind of a colloid is gum dammar? Preserve this emulsion for the subsequent experiment.

*Holmes, H. N., and Cameron, D. H. Emulsion. U. S. Patent 1,429,430. Dated Sept. 19, 1922.

Holmes, H. N., and Cameron, D. H. Gum dammar as an emulsifying agent. *Science*, 56, 724 (1922).

Expt. 23. Method of Determining the Phases of an Emulsion.—Several methods have been used to determine both the internal and external phases of an emulsion. For these tests use the two types of emulsions (Expts. 21, 22).

(*a*) *Palmer's internal phase method.*[7]—Using a glass rod, place a drop of an oil-in-water emulsion on a glass slide and cover with a cover

[7] Private communication from Dr. Leroy S. Palmer of this Division.

glass to prevent too much movement in the field. Observe under the microscope, focusing for the oil globule until the sharpest possible periphery is obtained. If the focus is *raised*, the globule shows a bright center before it disappears. Examine a water-in-oil emulsion in a similar manner, but *lower* the focus; the water globule will also show a bright center before it disappears. The success of these observations depends upon a good light and a sufficiently high-power microscope to obtain a fairly large globule.

Methods to determine the *external* phase are as follows:

(*b*) *Briggs' drop-dilution method.*—Using a glass rod, place a drop of the emulsion on a glass slide. Then place a drop of distilled water upon the drop of emulsion and stir the two together. Examine under a microscope. If the emulsified globules spread in the water, it is an emulsion of oil-in-water; but if there is no spreading, it is an emulsion of water-in-oil. This result can be checked by adding a drop of oil to a drop of emulsion and stirring as before. If the globules spread, the emulsion is one of water-in-oil; but if not, an emulsion of oil-in-water. An emulsion is diluted by adding more of the external phase.

(*c*) *Robertson's indicator method.*—Sprinkle a few particles of the red dye, Sudan III, upon a drop of the emulsion. Observe under the microscope. If the color spreads rapidly over the surface, it is an emulsion of water-in-oil; if, however, the color is confined to the globules of oil with which the particles are in actual contact, it is an emulsion of oil-in-water. Sudan III is readily soluble in oil, but insoluble in water; therefore the color cannot spread from the reddened oil globules to others in the drop because of the intervening water. This method is not as satisfactory as the drop method (*b*), but it is often very useful.

*Robertson, T. B. Notiz über einige Faktoren, welche die Bestandteile von Oel-Wasseremulsionen bestimmen. *Kolloid-Z.*, 7, 7–10 (1910).

*Newman, F. R. Experiments on emulsions. *J. Phys. Chem.*, 18, 34–54. Cf. 35 (1914). The Briggs' drop method is described.

Bhatnagar, S. S. Studies in emulsions. Part I. A new method of determining the inversion of phases. *J. Chem. Soc.*, 117, 542–552 (1920). An electrical conductivity method for determining the type of an emulsion is described.

Expt. 24. Inversion of Emulsions.—For the emulsions in this experiment, it is necessary to use olive oil which contains sufficient free oleic acid to form soap with all of the sodium hydroxide added. Equal volumes of the olive oil mixture and of a water solution must be used. Place 10 cc. of the olive oil mixture in each of 16 test tubes. To each of these tubes add a mixture, which consists of the volume of sodium hydroxide and of calcium chloride indicated in the table below, and

sufficient distilled water to make a total volume of 10 cc. Shake each tube vigorously. Determine the phases of the emulsions (Expt. 23 (*a*).

Vol. of 0.10 *M* NaOH in cc. used	Vol. of 0.10 *M* $CaCl_2$ in cc. used			
	0.25	0.5	0.75	1.0
1				
2				
3				
4				

Record the results in the table, using:

O–W = oil-in-water emulsion,
W–O = water-in-oil emulsion,
R = critical or inversion point.

If the experiment is successful, the results will show that when the ratio of sodium hydroxide to calcium chloride is greater than 4 : 1, an oil-in-water emulsion is produced; but when the ratio is less than 4 : 1, a water-in-oil emulsion is formed. However, when the ratio is exactly 4 : 1, neither type of emulsion predominates. This is called the *critical point.* At this point, there is in the system, one chemical equivalent of calcium chloride to two of sodium hydroxide, so that sodium oleate and calcium oleate are present in equivalent proportions. At this point also, the oil and water layers are characterized by a tendency to separate. The nature of the soap determines the type of the emulsion, sodium oleate causing the formation of an oil-in-water emulsion, and calcium oleate producing water-in-oil. The two types of emulsions may be roughly distinguished from each other by certain characteristics. An emulsion of oil-in-water has the consistency of milk, flows easily, and when shaken vigorously makes a metallic sound like that produced by water; while an emulsion of water-in-oil resembles butter, is somewhat viscous, and when shaken vigorously makes a sound like that produced by oil. Refer to Bancroft's explanation of the phenomena of this experiment.

Olive oil mixture.—Add sufficient free oleic acid to pure olive oil to make the oil 0.5 per cent with respect to the acid; then, add sufficient powdered Sudan III to saturate the mixture. Allow to stand over night, and filter through dry filter paper to remove any unused Sudan III.

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*Clowes, G. H. A. Protoplasmic equilibrium. *J. Phys. Chem.*, 20, 407–451 (1916).

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V. Emulsoids

Expt. 25. Ferric Arsenate Gel.—Place 100 cc. of 3 *N* ferric chloride solution in each of two beakers. To one add 75 cc., and to the other 50 cc. of *N* sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$, solution and stir thoroughly. Dialyze (Expt. 2) each mixture against distilled water, until the dialysate is free from chlorides. Explain how dialysis brings about gel formation.

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*Holmes, H. N., and Arnold, Rossleene. The peptization of ferric arsenate and phosphate and the formation of their gels. *J. Am. Chem. Soc.*, 40, 1014–1019 (1918).

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*Weiser, H. B., and Bloxsom, A. P. The formation of arsenate jellies. *J. Phys. Chem.*, 28, 26–40 (1924).

Expt. 26. Silicic Acid Gel.—Given a sodium silicate solution of sp. gr. 1.35–1.40, calculate the volume, which on dilution to 100 cc. with distilled water will give a solution having a sp. gr. 1.06. Prepare

such a solution, using freshly boiled distilled water. Place 50 cc. of a 0.5 *N* acetic acid solution in a beaker, and add, with constant stirring, the sodium silicate solution until the mixture is just alkaline to litmus; and then allow it to set. What are some of the principal factors upon which the setting of a silicic acid gel depends? Allow it to stand 12–48 hours. Is there a constant water of hydration in gels? What is syneresis? Pass a stream of carbon dioxide from a cylinder into 30 cc. of sodium silicate solution of sp. gr. 1.06 and observe whether a gel separates. Can a sodium silicate solution be used again and again for the preservation of eggs? What is Silica Gel and how is it used commercially?

van Bemmelen, J. M. Die Absorption. Das Wasser in den Kolloïden, besonders in dem Gel der Kieselsäure. *Z. anorg. Chem.*, 13, 233–356 (1896).

*Holmes, H. N. Silicic acid gels. *J. Phys. Chem.*, 22, 510–519 (1918).

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Expt. 27. Syneresis.[8]—Place about 10 cc. of a 2 per cent solution of unmilled rubber in benzene in each of three dry test tubes; since the solution is so viscous, estimate the amount needed by comparing it with a test tube containing 10 cc. of water. Add to each tube, a different volume of a mixture of sulfur monochloride and benzene as designated; immediately close with a cork, shake two or three times and allow to solidify.

(*a*) Add 10 cc. of the sulfur monochloride-benzene mixture.

(*b*) Mix 5 cc. of the sulfur monochloride-benzene mixture with 5 cc. of benzene and add.

(*c*) Mix 2.5 cc. of the sulfur monochloride-benzene mixture with 7.5 cc. of benzene and add.

Observe and record the time at which the different gels set; then note that soon after this, syneresis begins. Tap a tube with the hand and observe the vibration of the gel; this is a good illustration of a singing gel. Observe the gels after 18 or 24 hours. At this time, loosen

[8] The suggestion for this experiment was obtained from Dr. W. J. Kelly, Goodyear Tire and Rubber Company, Akron, Ohio.

those in tubes (*b*) and (*c*) from the sides, with a glass rod and note that syneresis is hastened. The rate of reaction depends upon the concentration of the sulfur monochloride. If a higher concentration of this reagent is used, syneresis will take place more quickly. This experiment also illustrates the principle of the vulcanization of rubber.

Sulfur monochloride-benzene mixture.—Add to 10 cc. of sulfur monochloride 90 cc. of benzene and mix thoroughly.

Holmes, H. N., Kaufman, W. F., and Nicholas, H. O. The vibration and syneresis of silicic acid gels. *J. Am. Chem. Soc.*, 41, 1329–1336 (1919).

Expt. 28. Sodium Chloride Gel.—Place in a *dry* test tube 10 cc. of thionyl chloride and add 1.5 gm. of powdered sodium salicylate, a small portion at a time, heating so that each will be dissolved before the addition of the next portion. Close the tube loosely with a cork, keeping it closed except at each addition of sodium salicylate. After cooling for an hour or two, a translucent gel, which shows a bluish fluroresence, results. If the tube is corked, the gel will keep for several weeks.

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*Karczag, L. Über kolloidale Metallachloride und Metallsulfate. *Biochem. Z.*, 56, 117–121 (1913).

Expt. 29. Dibenzoyl Cystine Gel.—Place in an Erlenmeyer flask 2 gm. of *l*-cystine (Expt. 102), suspending it in 100 cc. of distilled water; then add sufficient 10 per cent sodium hydroxide solution to dissolve the cystine, carefully noting the amount of sodium hydroxide used. Next add 10 gm. of benzoyl chloride and enough *additional* sodium hydroxide solution to make a total of 60 cc. of the latter. This keeps the solution alkaline. Shake the mixture vigorously until all the odor of benzoyl chloride has disappeared. Acidify the solution with hydrochloric acid. A stiff gel will result. Break this up by vigorous shaking and filter on a Büchner funnel. A layer of crystals of dibenzoyl cystine will remain on the filter. Wash these with distilled water. To recrystallize the dibenzoyl cystine suspend it in 85–90 per cent ethyl alcohol; dissolve it by heating on an electric hot plate, and then, while boiling, add sufficient hot distilled water to make a total volume of about 400 cc. Allow this to stand for about 24 hours; filter on a Büchner funnel, wash, and dry. Long silky needles, which melt at 180–181° C. (uncor.) are obtained.

Place 0.2 gm. of pure benzoyl cystine in a beaker, and dissolve it in 5 cc. of 95 per cent ethyl alcohol by heating on a hot plate. Add slowly 95 cc. of hot distilled water to the boiling solution. A slight

opalescence appears. Cover the beaker and allow to stand. In from 2 to 4 hours, a rigid transparent gel is formed. Allow the gel to stand several days. Observe and explain the changes which occur in it. The dibenzoyl cystine may be recovered if the freshly prepared gel is placed on a Büchner funnel and strong suction applied. The liquid filters through slowly and after 8 to 10 hours the dibenzoyl cystine remains practically quantitative on the filter.

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*Gortner, R. A., and Hoffman, W. F. An interesting colloid gel. *J. Am. Chem. Soc.*, 43, 2199–2202 (1921).

Wolf, C. G. L., and Rideal, E. K. The properties of dibenzoyl-cystine. *Biochem. J.*, 16, 548–555 (1922).

Expt. 30. Sol-gel Transformation: an Example of Hysteresis.—The properties of either a gelatin sol or gel depend upon its *previous history*, which includes several factors: first, the method of manufacture of the original gelatin; second, the time allowed for swelling; third, the temperature at which the sol was formed; and fourth, the length of time it was exposed to this temperature. Gelatin is an emulsoid which is easily reversible; that is

$$\text{Hydrosol} \rightleftharpoons \text{Hydrogel}$$

with change of temperature. In this transformation, the terms **setting point** and **melting point** are commonly used; the former being understood to mean the temperature at which the sol congeals, and the latter, that at which the gel liquefies. These temperatures, however, are not coincident as in the case of crystalline substances.

Place 15 gm. of air-dried gelatin in 85 cc. of distilled water contained in a flask, and allow to stand from 30 minutes to an hour. What colloidal phenomenon takes place during the soaking? To disperse the gelatin, place the flask in a hot water bath and stir continually until a homogeneous solution results. Determine the *setting* and *melting points* of the gelatin by the following methods: Use a 1000 cc. beaker as a water bath, place in it a stirring rod, and then cover with a triangular support, containing a center hole. In this insert a test tube, Fig. 6, and add 15–20 gm. of mercury so that it will stand perpendicular. Suspend a glass rod exactly in the center of the test tube with its end about 1 cm. above the mercury. Nearly fill the beaker with water at 15–20° C.; pour the gelatin sol into the tube until it is two-thirds full, and allow it to set. Then lift the rod with the test tube hanging to it about 3 cm. and clamp it. Raise the temperature of the bath slowly,

with constant stirring, until the test tube slides off the cylinder of the gel surrounding the rod and comes to rest on the support. This temperature is taken as the melting point. Lower the rod into its original position, remove the flame, and allow the bath to cool. Raise the rod slightly from time to time until it just lifts the test tube with it. This temperature is taken as the setting point. Make duplicate observations in each case. Define hysteresis as applied to colloid systems. How do the setting and melting points of agar-agar sols and gels compare with those of gelatin? The presence of salts affects the gelation temperature; some raise it, others lower it. How does the amount of water imbibed by gelatin from a saturated atmosphere compare with that imbibed when immersed in distilled water?

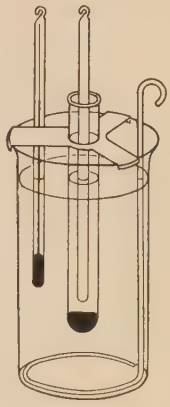

Fig. 6.

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Gortner, R. A., and Hoffman, W. F. Evidence of a structure in gelatin gels. *Proc. Soc. Expt. Biol. Med.*, 19, 257–264 (1922).

Expt. 31. Irreversible Gelation or Heat Coagulation.—To 10 cc. of egg albumin solution in a test tube, add an equal volume of distilled water and mix thoroughly. Place the tube in a water bath at 50° C. and then raise the temperature 1° C. a minute. Note carefully the temperature when definite turbidity appears; take this as the end-point of coagulation. Prepare four 10 cc. portions of the egg albumin solution and add to each respectively an equal volume of a 2, 6, 10, and 20 per cent sodium chloride solution; determine the coagulation temperature

in each case. What are the conclusions in regard to the coagulation of egg albumin in the presence of 1, 3, 5, and 10 per cent sodium chloride solutions? Chick and Martin found that heat coagulation of proteins consists of two processes, a denaturation or dehydration followed by agglutination or aggregation. Explain these processes.

Egg albumin solution.—Mix thoroughly one part of fresh egg white with one part of distilled water, then filter the solution to remove the precipitated ovoglobulin.

*Osborne, T. B., and Campbell, G. F. The protein constituents of egg white. *J. Am. Chem. Soc.*, 22, 422–450. Cf. 435–438 (1900).

Chick, Harriette, and Martin, C. J. On the "heat coagulation" of proteins. *J. Physiol.*, 40, 404–430 (1910).

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VI. Electrical Properties

Expt. 32. Cataphoresis.—The term cataphoresis is employed to denote the migration of a colloidal particle under the influence of an electrical current. Because of the fact that all colloidal particles possess an electrical charge, they are acted upon by the electrical current in much the same manner as if they were ions and consequently migrate through the liquid toward the pole possessing the sign opposite to that of the charge which they possess.

A convenient form of apparatus is illustrated in Fig. 7. It consists of a U-tube, the arms of which are about 15 cm. long and 1.3–1.5 cm.

in diameter. A thistle tube 3 or 4 mm. in diameter is fastened into the bottom of the U-tube, and is best at right angles to the plane of the tube. The arms of the U-tube are either graduated in millimeters throughout their length or a millimeter scale is placed behind them. Attached by rubber tubing to each arm of the U-tube are tubes, *A* and *B*, about 2 cm. in diameter, in which the electrodes are placed.

To fill the apparatus.—Fasten the apparatus rigidly in an upright position and place the electrodes in tubes, *A* and *B*. If the electrodes are of platinum or gold, use distilled water, or if zinc, use zinc sulfate, pouring either into tubes, *A* and *B*, until the connecting tubes are nearly filled. Close stopcock, *C*, and pour distilled water or preferably *a buffer solution* (Expt. 72) *which has the same hydrogen-ion concentration as the sol to be examined*, into the main U-tube until the liquid reaches about halfway up on the scale. The exact point depends upon the dimensions of the apparatus and should be determined by the instructor or student for each apparatus. Then fill the bulb, *E*, with the caramel solution (Expt. 57). Now open stopcock, *C*, slightly, so that the solution just seeps through. Allow the caramel solution to flow into the apparatus until the liquid rises above the inlets from tubes, *A* and *B*, and makes connection with the liquid already in those tubes. It is important to keep a sharp division between the caramel solution and the water layer or buffer solution in the main U-tube, and to prevent the two solutions from mixing. The volumes of solution employed must be such that when the apparatus is filled and ready for use the boundary line between the caramel solution and the buffer solution is somewhere near the center of the graduation scale. Without this it is impossible to measure accurately the rate or extent of migration.

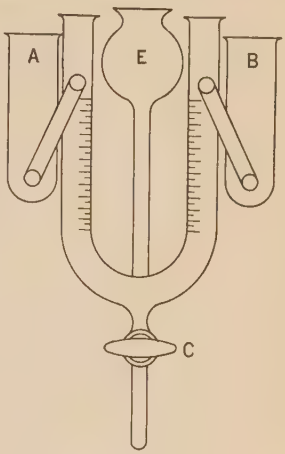

FIG. 7.

Connect the electrodes with a suitable source of *direct* electrical current, using a storage battery or the house current of 110 or 220 volts D.C. In case the conductivity of the sol and buffer solution is high, it may be necessary to insert a resistance in the circuit in order to control the amount of current flowing. *The exact condition for the best results must be determined for each apparatus and for each sol at the time the experiment is being conducted.* The use of zinc electrodes in zinc sulfate solution will prevent polarization of the electrodes. Platinum, gold or

silver electrodes may become polarized and thus decrease the efficiency of the apparatus. If they are used they should be made from strip metal or foil and should not be smaller than 2 sq. cm. area. The larger they are, the more rapid will be the rate of migration.

Turn on the current, and after a time, note that the position of the boundary between the caramel and the buffer solution has changed in relation to the graduations in the U-tube, having fallen in one arm and risen in the other. The migration is toward the pole of opposite sign to the charge on the caramel. Since the sign on the caramel is negative the migration will be toward the anode. A satisfactory rate of migration, 5–6 mm. per hour, will be obtained if the hydrogen-ion concentration of the caramel solution and the buffer solution is in the neighborhood of pH = 10–11. A lower pH will give a lower rate of migration. *The rate of migration becomes zero at the isoelectric point of the colloid particles.* This is one method of measuring the isoelectric point of colloid preparations. This experiment may be extended to include the measurement of the rate of migration at a series of hydrogen-ion concentrations, for the determination of the isoelectric point of the caramel particles.

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*Hauge, S. M. Studies in adsorption with carbons. Thesis for the degree of Doctor of Philosophy, University of Minnesota, December, 1926.

Svedberg, The., and Tiselius, A. A new method for the determination of the mobility of proteins. *J. Am. Chem. Soc.*, 48, 2272–2278 (1926).

Expt. 33. Electrical Endosmose or Electroendosmosis.—In Experiment 32 it was observed that the free electrically charged particles of a sol or gel migrated under the influence of an electric field. If the

colloidal particles are fixed as in a membrane or rigid gel, a similar relative displacement of the two phases results when a current is sent through the system. In such case the dispersion medium is transported in the direction of the electrode having *a charge of the same sign as the charge on the membrane.* This phenomenon is called electro-endosmosis or electroendosmose for it superficially resembles normal osmosis in that hydrostatic pressure is built up on one side of the membrane. Very early quantitative studies of electroendosmose showed:

(1) That the mass of liquid transported in unit time was directly proportional to the current strength.

(2) That the difference in hydrostatic pressure maintained between the two sides of the membrane, varies directly as the current strength; for a given membrane and constant current, it is directly proportional to the length, inversely to the sectional area of the membrane and proportional to the specific resistance of the liquid in the case of an aqueous solution.

(3) That for a given membrane, the difference in hydrostatic pressure is proportional to the applied potential.

A simple demonstration of electroendosmosis may be made with the apparatus shown in Fig. 8, Experiment 34. Assemble the apparatus as for electrodialysis, using one of the membranes suggested in that experiment. Fill each compartment two-thirds full of distilled water and add to each a small amount of acid or alkali. Connect with a source of D.C. current and observe the direction in which the water is transported. Measure the difference in water levels on each side of the membranes; or better, measure the amount of water transported, by drawing off the water, which accumulates in the end compartment, through a hole in the box placed at the original water level. If the latter method is used, add water to the opposite end compartment from time to time so that the flow may be continuous. Use as many different kinds of membranes as possible, and note the direction of flow in each case.

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Expt. 34. Electrodialysis.—The purification of sols and gels by the methods of dialysis has been noted already in Experiments 2, 3 and 14. However, it is sometimes desirable to carry out a dialysis more rapidly than is possible by the usual technic, as for instance, when

diffusion is the only factor operating to transfer the impurities through the membrane. There may also be instances where diffusion will not completely remove the electrolytes which are present. This holds for systems where the electrolytes are chemically bound to the colloid or where the forces of adsorption are very strong.

In such instances electrodialysis may be employed advantageously. It hastens the removal of *electrolytes* but does not affect appreciably the rate of dialysis of non-electrolytes. In principle, electrodialysis depends upon the transfer of electrolytes through the membrane under the influence of an imposed electrical current. A convenient apparatus is illustrated in Fig. 8. It consists of a water-tight box sawed into three sections, these being fitted together by dowels extending from one section into holes bored in the other sections. The partitions (*D*) and (*E*) are dialyzing membranes. These may be parchment paper or collodion films, or paper or cloth impregnated with collodion or with gelatin hardened by formaldehyde or potassium bichromate solution, or the membranes may be cut from a sheet of "Cellophane."[9] Should the migration of water due to electroendosmosis become a troublesome factor, the membrane adjacent to the cathode compartment may be of animal origin, such as goldbeater's skin or sheep skin or a bladder membrane.

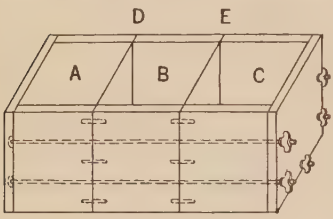

FIG. 8.

After the membranes are in place, draw up the joints between the various sections of the box tightly by means of bolts or clamps. Place the material to be dialyzed, for example 25–50 gm. of finely-ground agar or gelatin suspended in 1 liter of water, in the center compartment (*B*) and insert the electrodes in the end compartments (*A*) and (*C*). These may be a gold or platinum anode and a silver cathode or graphite plates which will serve for most purposes. Fill the electrode compartments with distilled water and connect the electrodes through an ammeter with a source of *direct current*. The voltage employed depends upon the concentration of electrolytes in the sol or gel under experiment. For sols or gels with a high content of electrolytes, the voltage should not exceed 55 volts at the beginning, but this may be increased to 110 volts or 220 volts as the current, as indicated by the ammeter, falls. The ions of the electrolyte migrate under the influence of the electric current, the cations collecting in the cathode (−) compartment and the

[9] "Cellophane" is transparent cellulose regenerated from "viscose" and manufactured by the DuPont Cellophane Company, Buffalo, N. Y. The 0.016″ thickness is admirably suited for this purpose.

anions in the anode (+) compartment. Changing the water in these compartments at intervals removes the electrolytes from the system. Continue the electrodialysis until the resistivity of the final portion of the distilled water in the anode and cathode compartments reduces the current, as measured in the ammeter, to approximately zero and until no appreciable change in current is produced by electrolytes migrating through the membranes. By the use of this apparatus it has been possible to remove practically all of the inorganic constituents, with the exception of silicon dioxide from agar and to reduce the ash content of gelatin to 0.01–0.02 per cent. However, it is obvious that inorganic constituents in colloidal form such as silicon dioxide are not eliminated by this procedure.

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Expt. 35. Coagulation of Suspensoid Sols by Electrolytes.—For many years it has been recognized that coagulation is an electrical phenomenon and that the particles of the sol are wholly or partially discharged during the coagulation process. This is brought about by the addition of electrolytes, whose ions carry a charge opposite in sign to that of the colloidal particles. The coagulating power of an electrolyte depends somewhat on the valency of its ions. It has also been found that the coagulating power of any given ion varies with the concentration of the colloidal solution. To determine the coagulating power of any electrolytic ion, add to a given volume of colloidal solution a quantity of electrolyte just sufficient to produce coagulation of the particles. The molar concentration, c, of the electrolyte in the total volume of the mixture is usually expressed in millimols per liter. As a result, the coagulating power of a given electrolyte on a given sample of colloid is expressed by $\frac{1}{c}$. The effect of mono-, di-, and tri-valent ions on both negatively and positively charged colloidal solutions is illustrated.

Place 18 cc. of an arsenious sulfide sol (Expt. 17) in each of five test tubes, and add to each respectively, 2 cc. of a 1.0 *M*, 0.8 *M*, 0.6 *M*, 0.4 *M*, and 0.2 *M* sodium chloride solutions. The latter may be prepared from the *M* sodium chloride solution by diluting the proper number of cubic centimeters with distilled water to make a total of 10 cc. Mix the contents of the five tubes by closing and gently reversing them two or three times; then allow to stand for an hour. In this manner determine the smallest amount of electrolyte which will cause the colloid to settle. If this procedure does not establish the concentration required, try others until the limit is established. Then calculate in millimols per liter the concentration of electrolyte necessary to cause coagulation, and from this determine its coagulating power. Note that the electrolyte is diluted by the sol to one-tenth of its original concentration. Repeat this experiment, using as electrolytes, a 0.1 *M* solution of barium chloride and a 0.001 *M* solution of lanthanum chloride, $LaCl_3$, $7H_2O$; dilute them in the same ratio as the sodium chloride solution.

In a similar manner determine the concentrations of sodium chloride, sodium sulfate, and trisodium phosphate solutions necessary to cause coagulation of ferric oxide sol (Expt. 14b). For this purpose begin with a 0.2 *M* sodium chloride solution, a 0.005 *M* sodium sulfate solution, and a 0.001 *M* trisodium phosphate solution. From the resulting determinations calculate their coagulating power.

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Browne, F. L. The heat of coagulation of ferric oxide hydrosol with sodium sulfate. *J. Am. Chem. Soc.*, 311–321 (1923).

Expt. 36. Mutual Coagulation of Suspenoid Sols.—Place in a series of 9 test tubes respectively, 1 cc., 2 cc., 3 cc., etc., of an arsenious sulfide sol (Expt. 17); then, keeping the same order, add respectively 9 cc., 8 cc., 7 cc., etc., of a ferric oxide sol (Expt. 14b). Gently invert each tube two or three times immediately after the addition of the second sol; then allow to stand for 15 minutes. Record the ratio of the sols in those tubes in which immediate coagulation occurs. If either sol is in great excess, no coagulation takes place. The sign of the charge of the mixture will be that of the sol present in excess; the charge of the other sol will be reversed. Explain the mechanism of mutal coagulation of suspensoid sols.

*Picton, H., and Linder, S. E. Solution and pseudo-solution. Part III. The electrical convection of certain dissolved substances. *J. Chem. Soc.*, 71, 568–573 (1897).

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*Billitzer, J. Theorie der Kolloide. II. *Z. physik. Chem.*, 51, 129–166 (1905).

Lottermoser, A. Beiträge zur Theorie der Koagulation der Hydrosole. *Kolloid-Z.*, 6, 78–83 (1910).

Bancroft, W. D. Report on peptisation and precipitation. Second report on colloid chemistry and its general and industrial applications. *Brit. Assoc. Advancement Sci. Reports.* pp. 2–16, 1919.

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Thomas, A. W., and Johnson, Lucille. The mechanism of the mutual precipitation of certain hydrosols. *J. Am. Chem. Soc.*, 45, 2532–2541 (1923); or Colloid symposium monograph. First national symposium on colloid chemistry. pp. 187–195. Department of Chemistry, University of Wisconsin, Madison, 1923.

Moore, W. Adherent arsenical preparations. *Ind. Eng. Chem.*, 17, 465–466 (1925).

Expt. 37. Coagulation of an Emulsoid Sol.—Suspend 20 gm. each of a patent and of a clear [10] wheat (*Triticum vulgare*) flour of high ash content, in 100 cc. of distilled water at 20° C. Extract for one hour at this temperature, shaking every 10 minutes to keep the flour particles

[10] The ash content of a flour is an index of flour grade. The percentage of ash in a high grade of "patent" flour is low; while in the less highly refined flours, produced simultaneously with the patent, the ash content increases, because in these flours there are large amounts of the outer portion of the wheat berry, which contain more mineral salts. Thus first "clear" and second "clear" flours are higher in ash content than patent, and second clear is higher than first clear.

in suspension. At the end of the hour, either filter on a dry filter paper or centrifuge until all the suspended matter has been thrown down, and decant the clear supernatant liquid. The water extract of a wheat flour contains a variety of soluble organic and inorganic substances. These include leucosin, a typical water-soluble protein which is coagulated by heat, and phosphates which are produced by the hydrolysis of phytin by phytase during extraction with water. Use two small Erlenmeyer flasks, and place in one 50 cc. of the patent flour extract and in the other a similar quantity of the extract of the clear flour. Boil the extracts for 5 minutes and filter. Explain the changes that take place in each. Add a drop or two of saturated sodium chloride solution to the patent flour extract after boiling, and explain the result.

Collatz, F. A., and Bailey, C. H. The activity of phytase as determined by the specific conductivity of phytin-phytase solutions. *J. Ind. Eng. Chem.*, 13, 317–318 (1921).

Bailey, C. H., and Collatz, F. A. Studies of wheat flour grades. I—Electrical conductivity of water extracts. *J. Ind. Eng. Chem.*, 13, 319–321 (1921).

*Bailey, C. H., and Peterson, Anna C. Studies of wheat flour grades. II—Buffer action of water extracts. *J. Ind. Eng. Chem.*, 13, 916–918 (1921).

VII. Diffusion in Gels

Expt. 38. Diffusion of Colored Substances.—Prepare about 150 cc. of a 15 per cent gelatin sol (Expt. 30). Fill six test tubes two-thirds full of the sol and allow to *solidify*. To each test tube add 5 cc. of one of the following substances: copper sulfate, Congo red, methylene blue (Expt. 56), red gold sol (Expt. 10), arsenious sulfide sol (Expt. 17), and either light green, acid violet, or acid green; and again allow to stand. At the end of 48 hours, measure in millimeters the diffusion distance of each substance. Explain the results. Is it possible to distinguish between colloidal and true solutions by this method?

Dye solutions.—Dissolve 0.25 gm. of a dye, such as Congo red, light green, acid violet, and acid green in 500 cc. of distilled water.

*Traube, J., und Shikata, M. Diffusion von Farbstoffen in Gele. *Kolloid-Z.*, 32, 313–316 (1923).

Expt. 39. Liesegang Rings.—The phenomenon of Liesegang rings was observed by its discoverer, for whom it is named, in 1896. He used a glass plate, coated with a gelatin gel containing a small amount of potassium dichromate, in the center of which he placed a drop of silver nitrate solution. The two molecularly dissolved substances diffused into each other, but the insoluble silver salt formed was not deposited in a continuous zone; instead there occurred a series of concentric rings, separated by apparently clear zones, the width of which increased

with the distance from the center. When the experiment is performed in a test tube, a periodic precipitation results.

Given a sodium silicate solution of sp. gr. 1.35–1.40, calculate the volume which on dilution to 100 cc. with distilled water will give a solution having a sp. gr. 1.06. Prepare such a solution and place 35 cc. in a large test tube; add an equal volume of acetic acid-potassium chromate solution; mix *quickly* by shaking two or three times for the gel sets in a few seconds. This gel is slightly basic in reaction. After the gel sets, cover it with a 0.5 *N* copper sulfate solution. Cork the tube and allow it to stand for several weeks, observing at frequent intervals. Give theories for this phenomenon.

When the experiment is performed in the following manner an artificial agate results: Prepare 200–250 cc. of silicic acid gel containing potassium chromate, according to the method described in the preceding paragraph, but allow it to set in a 250–300 cc. collodion bag (Expt. 1), so that the gel will have the shape of an onion. Tear off the collodion bag, place the gel in a large beaker, and cover it with 0.5 *N* copper sulfate solution. Allow this to stand for 5 or 6 weeks, keeping the gel covered with copper sulfate solution. Then pour off the solution, rinse the gel with distilled water, cut it in two and observe. The copper sulfate solution surrounds the gel on all sides and therefore diffuses concentrically into it; that is, diffusion takes place in three dimensions instead of in two as in the test tube.

Acetic acid-potassium chromate solution.—Add to 1000 cc. of 0.5 *N* acetic acid solution sufficient powdered potassium chromate to make the solution 0.2 *N*.

*Liesegang, R. E. "A-Linien." *Liesegang's Phot. Arch.*, 37, 321–326 (1896).

Bradford, S. C. Adsorptive stratification in gels. *Biochem. J.*, 10, 169–175 (1916).

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*Holmes, H. N. Experiments in rhythmic banding. *J. Am. Chem. Soc.*, 40, 1187–1195 (1918).

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Bradford, S. C. Adsorptive stratification in gels. IV. *Biochem. J.*, 14, 474–482 (1920).

Williams, A. M., and MacKenzie, Mary R. Periodic precipitation. Part I. Silver chromate in gelatin. *J. Chem. Soc.*, 117, 844–852 (1920).

Liesegang, R. E. Spezielle Methoden der Diffusion in Gallerten. Abderhalden, E. Handbuch der biologischen Arbeitsmethoden. Abt. III, B, Heft 1. S. 33–130. Urban und Schwarzenberg, Berlin, 1922.

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Davies, E. C. H. Liesegang rings. II. Rhythmic bands of dyes on filter paper and cloth by evaporation. The refractivity, surface tension, conductivity, viscosity, and Brownian movement of dye solutions. *J. Am. Chem. Soc.*, 44, 2705–2709 (1922).

Bhatnagar, S. S., und Mathur, K. K. Studien über Banstrukturen. Die Synthese gebänderer Steine. *Kolloid-Z.*, 30, 368–371 (1922).

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McGuigan, H., and Brough, G. A. Rhythmic banding of precipitates (Liesegang's rings). *J. Biol. Chem.*, 58, 415–423 (1923).

Dhar, N. R., and Chatterji, A. C. Studies on the formation of periodic precipitates. II. *J. Phys. Chem.*, 28, 41–50 (1924).

Expt. 40. Formation of Lead Iodide Crystals in a Gel.—Place in a large test tube 35 cc. of acetic acid-lead acetate solution, and add an equal volume of sodium silicate solution of sp. gr. 1.06 (Expt. 39); mix thoroughly and allow to stand over night so that the gel may set firmly. Then cover it with 2 *N* potassium iodide solution. Cork the tube and allow to stand several weeks, observing from time to time. Since this concentration of the potassium iodide solution has a greater osmotic pressure than the gel system, the reaction must take place within, rather than above the gel.

Acetic acid-lead acetate solution.—Add to 1000 cc. of *N* acetic acid solution sufficient lead acetate to make it 0.04 *N* with respect to the latter substance.

Hatschek, E. Reaktionen im Kieselsäure-Gel. *Kollid-Z.*, 10, 77–79 (1912).

Simon, A. Bleibaum im Kieselsäure-Gel. *Kollid-Z.*, 12, 171 (1913).

*Holmes, H. N. The formation of crystals in gels. *J. Franklin Inst.*, 184, 743–773 (1917).

Hubbard, G. D. Colloids in geologic problems. *Am. J. Sci.* [5], 4, 95–110 (1922).

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VIII. Ultrafiltration

Expt. 41. Ultrafiltration of a Gold Sol.—The term *ultrafiltration* has been used by Bechhold to describe the separation of the disperse phase of a sol from its dispersion medium by filtration, under pressure, through a porous membrane impregnated with a gel. This method also provides a means for determining the size of particles in colloidal solution by the use of ultrafilters of definite and varying permeability.

Prepare two collodion bags (Expt. 1), using an Erlenmeyer flask, the diameter of whose base is slightly greater than that of the Büchner funnel subsequently employed for the filtration. Immerse one bag in distilled water and *harden* the other (Expt. 3). Using a gold sol (Expt. 10), compare the degree of permeability of the two bags by the following method:

(*a*) Carefully fit a filter paper into a 7.5 cm. Büchner funnel; moisten with distilled water; place on it the unhardened collodion bag, containing 50–75 cc. of the gold sol; and apply suction until the volume of the original solution has been reduced to about 25 cc.

(*b*) Repeat the above process, using the hardened collodion bag. Now compare the color of this sol with the original. Compare the filtrates from (*a*) and (*b*) and explain the results. If there is no difference between the filtrates, it is due to the preparation of the untreated collodion bag, which sometimes results in a membrane with pores too small to permit the passage of the colloidal particles, it thus becomes an ultrafilter as effective as the hardened collodion bag.

The method of ultrafiltration may also be used for the purification and concentration of enzyme solutions, provided a collodion of definite composition is employed for making the membranes. Such collodion membranes are impermeable to enzymes, but are permeable to water and to the highly dispersed impurities present in the enzyme solutions. If a solution is purified by any other method, it may still be concentrated by ultrafiltration.

Preparation of ultrafilter for enzyme solutions.—Pour about 100 cc. of collodion into a 2-liter measuring cylinder; cover the end with the hand and incline and rotate the cylinder to secure a uniform distribution of the collodion on its walls; then gradually pour any excess back into the collodion bottle; invert and dry for 10 minutes. Next carefully fill the cylinder with water and, after allowing it to stand 10 or 15 minutes, pour it out. Cautiously loosen the collodion membrane around the mouth of the cylinder with the tip of a knife blade, and pour about 100 cc. of distilled water between the collodion film and the walls of the cylinder. Again rotate the cylinder gently so that the water separates all of the collodion film from the glass; then draw it out. Fill the bag with water, wipe dry on the outside, and examine for holes. Empty the water, cut the membrane longitudinally, and spread it out flat upon a piece of wet filter paper. Do not allow the membrane to dry or it will become impermeable. Cut a circular piece 15–20 cm. in diameter. Upon it place concentrically a Graham dialyzer or a 1000 cc. Erlenmeyer flask from which the bottom has been cut and the edges ground smooth. Fold the edge of the membrane up against the sides of the

dialyzer or flask, cementing it firmly with collodion containing 4 parts of ether to 1 of alcohol. Apply the cementing collodion with a small paint brush; also coat most of the outside of the dialyzer or flask as well as the edge of the membrane. Place 3 or 4 thicknesses of wet filter paper in a 12.5 or 15 cm. Büchner funnel, and set upon it the dialyzer fitted with the collodion membrane. Pour either melted paraffin or vaseline between the dialyzer and the sides of the funnel to the depth of 2.5 cm. or more, so as to make an air-tight seal. Ultrafiltration may be carried on continuously by the use of a constant level siphon for supplying the enzyme solution; however, it is necessary to insert a stirrer to keep the solution thoroughly agitated. Figure 9 illustrates the completed ultrafiltration apparatus. When not in use the ultrafilter must be kept covered with water to which a preservative, such as toluene or thymol, has been added. Reynolds recommends the use of a 1 : 2000 solution of chinosol (potassium oxyquinoline sulfonate).

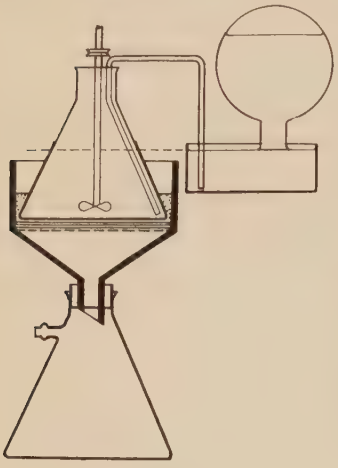

Fig. 9.

Collodion for enzyme ultrafilter.—Dissolve 5 gm. of Anthony's snowy cotton [11] in a mixture of 50 cc. of 95 per cent ethyl alcohol, 50 cc. of U.S.P. ether, and 5 cc. of glacial acetic acid.

Martin, C. J. A rapid method of separating colloids from crystalloids in solutions containing both. *J. Physiol.*, 20, 364–371 (1896).

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Walpole, G. S. Notes on collodion membranes for ultrafiltration and pressure dialysis. *Biochem. J.*, 9, 284–297 (1915).

Kober, P. A. A new form of ultra-filter: Some of its uses in biological and synthetic organic chemistry. *J. Am. Chem. Soc.*, 40, 1226–1230 (1918).

*Reynolds, F. W. The rapid analysis of sugars. Purification and concentration of enzyme solutions. *Ind. Eng. Chem.*, 16, 169–172 (1924). This includes

[11] This material may be obtained from Ansco Photoproducts, Binghamton, New York.

a description of the concentration of enzyme solutions by means of a collodion ultrafilter.

Paine, H. S., Badollet, M. S., and Keane, J. C. Colloids in Cane and Beet Sugar Manufacture. *Ind. Eng. Chem.*, 16, 1252–1258 (1924).

IX. Imbibition

Expt. 42. Swelling of Soft Rubber.—Cut a strip of dental sheet rubber (Expt. 101) about 10 × 2 cm. and slit it lengthwise almost to the end. Fill a short test tube nearly full of either chloroform, ether or benzene; hang the divided rubber strip so that one half is outside, and the other half inside the test tube. After a few minutes, compare the halves. Remove the rubber strip from the test tube and expose to the air for half an hour. Explain the results.

Flack, M., and Hill, L. The imbibition of rubber. *J. Physiol.*, 40, xxxiii (1910).

Expt. 43. Heat of Hydration.—Prepare practically anhydrous commercial corn starch by drying it for 48 hours in an oven at 100° C., and then keep in a desiccator until ready for use. Allow 10 gm. of the dry starch contained in a weighing bottle, a Dewar vacuum tube 120 × 45 mm., and a bottle of distilled water to stand until they reach the same temperature; then place the starch in the vacuum tube and quickly add 10 cc. of the distilled water. Stir immediately with a thermometer graduated to 0.10° C. and observe the change in temperature. Make another determination using air-dry starch. Compare the results from the two determinations. About how much moisture does air-dry starch contain? Why is very dry starch used in the preparation of baking powder?

*Rodewald, H. Über die Quellung der Stärke. *Landw. Vers.-Sta.*, 45, 201–227 (1895).

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Patten, H. E. Energy changes accompanying absorption. *Trans. Am. Electrochem. Soc.*, 11, 387–407. Cf. 397 (1907).

Posnjak, E. Ueber den Quellungsdruck. *Kolloidchem. Beihefte*, 3, 417–456 (1912).

Daniels, F., Kepner, B. H., and Murdick, P. P. The heat of hydration and specific heat of wheat flour. *J. Ind. Eng. Chem.*, 12, 760–763 (1920). This article contains a description of the method for calculating the number of calories evolved.

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Anderson, M. S. The heat of wetting of soil colloids. *J. Agr. Research*, 28, 927–935 (1924).

Expt. 44. Effect of Acid and Alkali on Fibrin.—Place 5 gm. of powdered fibrin [12] in a tall 200–300 cc. beaker, add 150 cc. of distilled water and allow to stand for about 2 hours. Then add 3 cc. of a 10 per cent potassium hydroxide solution; this is sufficient to give a concentration of 0.2 per cent. Stir thoroughly during this addition and for a few minutes thereafter, and then allow the mixture to stand an hour. Explain the change that takes place. Neutralize the mixture with 3 cc. of 10 per cent hydrochloric acid, added very slowly and with vigorous stirring. The protein should now be at its isoelectric point. Next add 3 cc. of 10 per cent hydrochloric acid; this is sufficient to give a concentration of 0.2 per cent. Explain the result obtained. Again neutralize the mixture, using potassium hydroxide, and observe the result. What is the effect of the presence of the potassium chloride formed in the reaction? What is the isoelectric point? What are the methods used for its determination? State some of the theories of imbibition.

*Fischer, M. H., and Moore, Gertrude. On the swelling of fibrin. *Am. J. Physiol.*, 20, 330–342 (1907–08).

Donnan, F. G. Theorie der Membrangleichgewichte und Membranpotentiale bei Vorhandensein von nicht dialysierenden Elektrolyten. *Z. Electrochem.*, 17, 572–581 (1911).

Donnan, F. G., and Harris, A. B. The osmotic pressure and conductivity of aqueous solutions of Congo-red and reversible membrane equilibria. *J. Chem. Soc.*, 99, 1554–1577 (1911).

Proctor, H. R. The equilibrium of dilute hydrochloric acid and gelatin. *J. Chem. Soc.*, 105, 313–327 (1914).

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*Bosworth, A. W. Fibrin. *J. Biol. Chem.*, 20, 91–94 (1915).

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Robbins, W. J. An isoelectric point for plant tissue and its significance. *Am. J. Botany*, 10, 412–439 (1923).

[12] To secure electrolyte-free fibrin, it may be necessary to wash commercial fibrin. In this case place the fibrin in a 500 cc. Erlenmeyer flask, add 450 cc. of distilled water and enough toluene to saturate it; shake occasionally during 24 hours, then decant the supernatant liquid. Wash the residue several times in this manner and dry.

Expt. 45. Action of Dilute Acids on Wheat Gluten.—Place 150 gm. of " strong " wheat (*Triticum vulgare*) flour [13] in a bowl or other suitable container and add distilled water gradually, while stirring, until a stiff dough is formed. This will require about 60 cc. of water per 100 gm. of flour. Knead the dough thoroughly and allow it to stand under distilled water for an hour. Why is it placed under water? Next wash in a stream of distilled water at room temperature for 15–20 minutes to remove the starch. Using glass plates, press out the gluten which remains into a thin sheet about 3 mm. thick. After standing about 5 minutes, remove it to a wet board and cut into discs with a large cork borer. Place these in distilled water and allow to stand for about 20 minutes so that they may take up any water which may have been pressed out by the pressing and cutting processes. Next place the discs on an inclined perforated porcelain plate and drain for *exactly* 7 minutes. To prevent loss of moisture by evaporation, quickly weigh a disc to the nearest centigram and then place it in 100 cc. of one of the different concentrations of the *assigned acid*; proceed thus until a disc has been added to each concentration. Use *N* solutions of hydrochloric, sulfuric, acetic, orthophosphoric, lactic, oxalic, tartaric, citric, and formic acids, diluting each to the following concentrations: 0.002 *N*, 0.004 *N*, 0.01 *N*, 0.02 *N*, 0.04 *N*, 0.1 *N*, 0.2 *N*, 0.5 *N*. Students should work in pairs, each pair using a different acid. Allow the disc to remain in the acid for *exactly* 50 minutes, remove them and drain 7 minutes as before, then weigh. Calculate the results in grams, on the basis of the increase in weight per gram of moist gluten. Plot a curve on coordinate paper, using the normality of the acid as abscissae, and the gram water imbibed, per gram of moist gluten, as ordinates.

From the normalities of lactic, acetic, orthophosphoric, hydrochloric, and oxalic acids, Sharp and Gortner have determined their corresponding hydrogen-ion concentrations or pH values (Expt. 72) at 25° C. These results are recorded in Table I. Pure solutions were used for the determinations; however, this does not mean that the hydrogen-ion concentration of the liquid in actual contact with the disc remained the same during the period of imbibition.

Students, who have used any acid given in Table I, should plot a

[13] In differentiating between the qualities of wheat flour, the terms "strong" and "weak" have been generally accepted. The former designates the flours milled from the hard spring wheats, particularly those from the northern portion of the Great Plains area, which have superior baking qualities; while the latter refers to the flours milled from the softer wheats, which have inferior baking qualities; gluten can be washed from a "strong" wheat flour much more readily than from a "weak" flour.

TABLE I

Acid	Normality	pH
Lactic	0.5	1.89
	0.2	2.11
	0.1	2.27
	0.04	2.49
	0.02	2.67
	0.01	2.82
	0.005	3.00
	0.002	3.23
Acetic	0.5	2.50
	0.2	2.69
	0.1	2.85
	0.04	3.08
	0.02	3.24
	0.01	3.39
	0.005	3.54
	0.002	3.76
Orthophosphoric	0.5	1.33
	0.2	1.59
	0.1	1.79
	0.04	2.05
	0.02	2.26
	0.01	2.49
	0.005	2.73
	0.002	3.08
Hydrochloric	0.5	0.46
	0.2	0.81
	0.1	1.09
	0.04	1.46
	0.02	1.77
	0.01	2.06
	0.005	2.35
	0.002	2.73
Oxalic	0.5	1.03
	0.2	1.29
	0.1	1.52
	0.04	1.82
	0.02	2.10
	0.01	2.36
	0.005	2.62
	0.002	3.00

curve on the same coordinate paper used before, with the pH of the acid as abscissae, instead of the corresponding normality.

HOFMEISTER, F. Zur Lehre von der Wirkung der Salze. Fünfte Mittheilung. Untersuchungen über den Quellungsvorgang. *Arch. Expt. Path. Pharmakol.*, 27, 395–413 (1890).

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SHARP, P. F., and GORTNER, R. A. Physico-chemical studies of strong and weak flours. II. The imbibitional properties of the glutens from strong and weak flours. *J. Phys. Chem.*, 26, 101–136. Cf. 128–133 (1922).

X. Viscosity and Plasticity

A liquid is generally defined as a substance which has no elasticity of shape and which offers no resistance to a shearing force. In the above definition it is assumed that an ideal liquid would offer no resistance to a shearing force. Actually all liquids and even gases do manifest a certain resistance to the motion of their molecules over each other. This internal friction or resistance to flow is called viscosity. The reciprocal of viscosity is called fluidity.

The unit for viscosity measurement in the C.G.S. system is defined as that force which when exerted on unit area between two parallel planes 1 cm. square and placed 1 cm. apart would produce a difference in velocity of streaming of the two planes of 1 cm. per second.

It was suggested by Delley and Parr and further recommended by Bingham and Jackson that this unit be called the " poise " in honor of Poiseuille's fundamental work on viscosity. It is frequently more convenient to use a submultiple of this unit which therefore may be called properly the " centipoise." It so happens that one centipoise is almost exactly the absolute viscosity of water at 20° C., the correct value being 1.0050 centipoise. Viscosity expressed in terms of the above units should be called absolute viscosity. The viscosity of a liquid is frequently expressed in terms of the viscosity of another liquid used as a standard. It is then called specific viscosity. It is better, wherever

possible, to express results in terms of absolute viscosity units. An apparatus used for the measurement of viscosity is called a viscometer. A great number of these have been devised, and they may be classified under two general heads: first, those in which the flow of liquid through capillary tubes is determined; and second, those in which the movement of some object in the fluid is determined.

(1) *Capillary tube viscometers.* These instruments are generally provided with a reservoir and receiving bulb connected by means of a capillary glass tube. The time required for the liquid to flow from the reservoir through the capillary into the receiving bulb is determined by the means of a stop watch. The additional information necessary for calculating the viscosity is the length of the capillary, its diameter, the volume flow, the force producing the flow which includes the hydrostatic head of the liquid, and its density. In addition to the hydrostatic head of the liquid, compressed air may be used in forcing the liquid through the capillary. If compressed air is used, the magnitude of this force must be known exactly and added to the hydrostatic pressure. In accurate work, corrections should be made for the kinetic energy unexpended in the flow through the capillary and for the friction against the parts of the instrument other than the capillary. As a general thing it is more convenient to standardize the viscometer by means of a liquid whose viscosity is accurately known rather than to attempt to calculate the viscosity from the dimensions of the instrument. The Ostwald viscometer is the simplest of the capillary tube instruments. In this, however, the liquid flows only by the force of its hydrostatic head. In making a comparative viscosity determination of two liquids with the Ostwald viscometer, equal volumes should be used. The viscosities of two liquids are directly proportional to their densities multiplied by their rates of flow as determined by means of a stop watch. The viscosity (ηx) may be calculated from the following equation:

$$\eta x = \frac{\eta s \; tx \; Dx}{ts \; Ds}.$$

Where ηs is the viscosity of the standard at the temperature of the determination, tx and ts, respectively, are the times of flow of the unknown and standard and, Dx and Ds, are the densities.

The absolute viscosities and densities of a number of water-sucrose solutions taken from the work of Bingham and Jackson are given in Table II:

TABLE II

VISCOSITY IN CENTIPOISES AND DENSITIES OF SUCROSE SOLUTIONS CONTAINING 0, 20, 40, AND 60 PER CENT SUCROSE BY WEIGHT

Temperature	0 Per Cent		20 Per Cent		40 Per Cent		60 Per Cent	
	Viscosity	Density	Viscosity	Density	Viscosity	Density	Viscosity	Density
15°	1.141	0.9991	2.267	1.0823	7.468	1.1784	74.6	1.2888
20°	1.005	0.9982	1.960	1.0809	6.200	1.1765	56.5	1.2864
25°	0.894	0.9971	1.704	1.0794	5.187	1.1744	43.86	1.2840
30°	0.802	0.9957	1.504	1.0777	4.382	1.1721	33.78	1.2814
40°	0.653	0.9923	1.193	1.0737	3.249	1.1676	21.28	1.2762

(2) *Torsion wire viscometer.* The MacMichael viscometer may be taken as an example of an instrument of this type. The viscosity of a solution is measured by the torsion produced on a wire, one end of which is rigidly fixed; the other end is attached to a disc, or cylinder, and suspended in a metal cup containing the liquid being investigated. The metal cup is rotated at constant speed by a motor, thus producing a torque on the wire, the magnitude of which is measured on a dial scale, rigidly attached through a spindle to the disc. If the cylinder is used, provided the effect of the ends is eliminated, it is possible from the dimensions of the instrument and the speed of rotation of the cup to calculate with a fair degree of accuracy the viscosity of the liquid. It is much easier with these instruments to calibrate them with liquids whose viscosities are known.

Within an ordinary range, using a capillary viscometer the various rates of flow obtained with a given pure liquid at a given temperature, subjected to different pressures, are inversely proportional to the pressures used; that is, the viscosity of a liquid itself is a constant, independent of the rate of shear. The above relation was discovered by Poiseuille and is usually referred to as Poiseuille's law. This law holds for pure liquids, molecular solutions, and probably dilute suspensoid sols provided there is no instrument effect; it does not hold for the more concentrated sols. The apparent viscosity of sols in which the dispersed phase occupies a considerable portion of the total volume, decreases markedly with increasing rates of shear. The viscosity of colloidal systems is always greater than that of the pure dispersion medium. Changes in colloidal systems which are hardly perceptible by any other

method are easily followed by changes in viscosity. The importance of viscosity measurements for the study of colloids so impressed itself upon Graham that he called the viscometer a "Colloidiscope."

Equations, based on more or less theoretical grounds, have been developed by a number of investigators to express the viscosity of colloidal systems in terms of the viscosity of the dispersion medium and some relationship with the volume occupied by the disperse phase. The viscosity of colloidal solutions increases as the relative volume occupied by the colloidal particles increases. In the case of an emulsoid sol the volume occupied by the colloidal particles is not the volume of the material in the dry state, but some value very much greater because imbibition or hydration takes place in these materials. Thus viscosity may be used to measure the relative degree of imbibition or hydration of emulsoid colloids.

The viscosity of a molecular solution is defined by the concentration and temperature: that of a suspensoid includes also another factor, the degree of dispersion; and Ostwald lists ten factors which may influence the viscosity of an emulsoid, namely, (1) concentration, (2) temperature, (3) degree of dispersion, (4) solvate formation or hydration, (5) electric charge or ionization, (6) previous thermal treatment, (7) previous mechanical treatment, (8) inoculation with small quantities of more viscous colloids, (9) time, and (10) the addition of both electrolytes and non-electrolytes. This list should include a factor, most important from the standpoint of the more concentrated colloidal systems, that is the rate of shear.

Bingham made an important contribution to the study of colloid systems by establishing the fundamental differences between viscous and plastic flow. If a substance is viscous, theoretically, a flow will be produced no matter how low the rate of shear, provided the shearing force acts over a sufficient interval of time; such a substance is a true liquid. A plastic substance on the other hand will not flow until a certain definite shearing force has been exceeded; such a substance should be classified as a plastic solid. This minimum shearing stress which must be exceeded before flow will start has been called the "friction constant" or the "yield value" of the plastic substance. The differences between the two types of flow are perhaps brought out more clearly by Chart 1, in which the volume of flow through a capillary tube, in cubic centimeters per second is plotted against the pressure causing the flow expressed in grams per square centimeter. The line A represents the results obtained with a pure liquid while the line B represents the results obtained with a plastic substance. The intersection of the extension of the straight

portion of the line B, as shown by the broken portion, to the pressure axis represents the yield value. The fact that some flow is obtained at pressure below this value has been attributed to seepage. It can be seen from this figure that the way to distinguish between a very viscous liquid and a plastic solid is to determine the rates of flow for at least two different pressures and plot the results. If the resulting line passes through the origin of the axis, the substance is a liquid and is viscous, but if the line intersects the pressure axis at some definite value, the substance is a plastic solid.

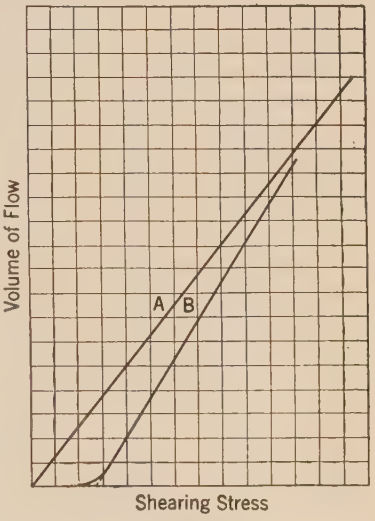

CHART 1.

The dispersed phase must exceed a certain minimum value before the system becomes plastic; below this value the system is viscous. There is a linear relationship between the fluidity and the concentration of a suspension up to the point where the system ceases to be viscous and becomes plastic. The viscosity and plasticity of colloidal solutions are apparently not entirely independent of the instrument in which they are measured. Flocculation and deflocculation have a very marked effect on the plasticity of a system.

From the figure it is apparent that two values are required to express the plasticity of a solid. (1) The "yield value" representing the pressure which must be exerted before the theoretical flow will start and (2) the "mobility" or its reciprocal the "rigidity"; these terms correspond to the "fluidity" and "viscosity" of a liquid and are determined from the straight portions of the curve.

The equation for the determination of the rigidity differs from that for viscosity only by the introduction of the factor $(P-p)$:

$$\text{Rigidity} = \frac{Gr^4t(P-p)}{8V(l+\lambda)} - \frac{1.12\rho V}{8\pi t(l-\lambda)}.$$

Where r is the radius of the capillary, l its length, λ a correction added to the length for friction outside of the capillary, t is the time of flow in seconds, V the volume in cc., G the gravitational constant, P the pressure in grams per square cm., p the yield value expressed in grams per square cm., and ρ the density.

The second part of the equation is a correction for the kinetic energy of the issuing flow and is generally assumed in plasticity studies to be zero. The correction λ is also generally assumed to be zero. Let $V/t = q$ the flow in cc. per sec., then the equation as simplified can be grouped:

$$\text{Rigidity} = \frac{\pi G r^4}{8l}\frac{P-p}{q},$$

and as,

$$\frac{\pi G r^4}{8l} = K,$$

for a given capillary, we may write the equation as follows:

$$\text{Rigidity} = \frac{K(P-p)}{q}.$$

The value of K may be determined from the dimensions of the capillary or by means of a viscous liquid whose viscosity is of about the same order of magnitude as the rigidities to be determined. Thus

$$K = \frac{q\eta}{P}.$$

where η is the viscosity of the standard liquid used. It has been shown that in reality the plasticity equation is not as simple as the original equation of Bingham would suggest, the values which it yields being approximate.

Kelly has recently developed an improved formula for the calculation of plasticity:

$$\frac{\Delta v}{\Delta t} = \phi\frac{\pi P R^4}{8\left(\frac{l_2 + l_1}{2}\right)}.$$

Where Δv is the volume in cc. between successive graduations, ϕ is the fluidity constant, P is the pressure applied, R is the average radius of the section of the capillary of length l_2, l_1 is the distance from the end of the tube in centimeters, to the first graduation, l_2 is the length l_1 plus the first calibration interval, and Δt is the time of flow between successive graduations.

Expt. 46. Apparent Viscosity and Plasticity of Wheat Flour-in-water Suspensions.—(*a*) *Viscosity as a measure of the hydration capacity of wheat flour.*—In this experiment either a torsion wire viscometer or an Ostwald viscometer, which has a bulb with a capacity of about 20 cc. and a capillary whose radius is about 1.5 mm., may be used. Weigh out 20 gm. of flour; make up to a total volume of 100 cc. with distilled water, and allow to stand with occasional shaking for one hour. If

the MacMichael viscometer is used, pour the entire 100 cc. into the cup and make four readings. Then make readings after the addition of the following increasing amounts of normal acids or alkalies: 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00, 8.00 and 10.00 cc. If the Ostwald viscometer is used, introduce only 25 cc. of the mixture and make duplicate readings, determining the time by means of a stop watch after the addition of the following amounts of normal acids or alkalies: 0.00, 0.10, 0.20, 0.30, 0.40, 0.50, 0.80, 1.00, 1.50, 2.00 and 3.00 cc. Both instruments may be calibrated with standard solutions, for example a 60 per cent sucrose solution which has a viscosity of 43.86 centipoise at 25° C. It must be remembered that this mixture is really plastic and consequently it requires two constants to express its flow. The results obtained will, however, show the marked effect of the added solutions on the imbibition of the flour proteins. For comparative purposes as many different acids as possible should be used by the various students.

(*b*) *Effect on imbibition of the salts present in wheat flour.*—It has been shown by numerous workers that salts exert a marked inhibiting action on imbibition of emulsoid colloids, in the presence of either acids or alkalies; also, that the water extract from wheat flour contains a certain amount of dissolved electrolytes which would exhibit an inhibiting effect on imbibition. Bailey and Collatz found that the soluble electrolyte content of a water extract of wheat flour as measured by conductivity was related to the ash content of the flour, therefore, the viscosity of a low grade flour would be depressed more than the viscosity of a high grade flour. This is due to the difference in soluble electrolyte content. Thus the imbibitional strength of the proteins would be masked to different degrees in the different grades of flour. This masking effect of salts present in the original flour is more apparent in the case of imbibition with acids than with alkalies.

Shake 20 gm. of a sample of the same flour used in the previous viscosity measurements, with about 100 cc. of distilled water and then add about 900 cc. more. Shake at 10-minute intervals during 45 minutes and then let stand 15 minutes. Decant the supernatant liquid as completely as possible; a residue of about 75 cc. will remain. Add 500 cc. of distilled water to this residue, shake, let stand 15 minutes, decant the supernatant liquid and make the residue up to a total volume of 100 cc. with distilled water. Determine the viscosity of this mixture according to the method previously used, making measurements after the addition of the following total amounts of acid in the case of the MacMichael viscometer: 0.00, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.80, 1.00, 1.50, 2.00, 3.00, 5.00 and 10.00 cc. Use 0.05, 0.10, 0.15, 0.20,

0.25, 0.30, 0.40, 0.60,0 .80, 1.00, and 2.00 cc. if the Ostwald viscometer is employed.

(*c*) *Plasticity of flour suspensions.*—Take a weighed amount of flour ranging from 60 to 80 gm. and make up to a total volume of 200 cc. with distilled water at a temperature of 25° C. Rotate the flask to produce a suspension and place in a water thermostat for 30 minutes. Pour the mixture into a 500 cc. Erlenmeyer flask and shake in a mechanical shaker for exactly 1 hour. Remove the flask from the shaker and quickly determine the rate of flow of four different samples of the mixture, using various pressures to produce the flow. The time of flow should be determined by the use of a stop watch. Plot the volume of flow expressed in cubic centimeters per second against the pressure in grams per square centimeter and from the extension of the straight portion of the line to the pressure axis read the yield value. The constant K of the simplified plasticity equation may then be determined by either of the two methods given. The radius of the capillary in centimeters may be determined approximately by filling the capillary with mercury, measuring the length of the mercury thread and weighing the mercury. From the density of mercury which may be taken as 13.546 at 20° C. and 13.534 at 25° C. the radius of the capillary may be calculated. Having found the constant K and having determined p, calculate the rigidity of the mixture as shown by each of the determinations which fall along the straight portions of the curve.

A simple plastometer may be constructed by using a glass tube about 2.2 cm. internal diameter and 10.0 cm. length. Close the bottom end with a rubber stopper through which is inserted the end of a glass capillary tube about 10 cm. in length and about 0.08 cm. internal diameter. Close the upper end by means of a rubber stopper through which is inserted a glass tube for the admission of the air pressure. In order to keep these stoppers in place when the air pressure is turned on small blocks of wood with holes for the passage of the capillary and the air-pressure tube respectively should be placed, one on the top and the other on the bottom, and connected by means of long bolts. Such a plastometer should easily withstand pressure of over 1 meter of mercury and may be readily taken apart for cleaning and refilling. It is essential that the pressure under which the flow is taking place should remain constant during the determination. This may be accomplished by first filling a metal pressure reservoir of several liters capacity with air at the pressure it is desired to use in causing the flow. The pressure can be measured by means of a mercury manometer. The air pressure in the reservoir can be obtained by means of an air pump of any kind or from the compressed air line which is part of the desk equipment. Con-

nect the plastometer with the pressure reservoir by means of a two-way stopcock, one side connecting the plastometer to the air and the other, to the pressure reservoir. For accurate work the whole plastometer should be immersed in a water thermostat. The plastometer described will hold a little over 25 cc. An illustration of the apparatus may be found in Cereal Chemistry, Vol. III, p. 48. The material issuing from the capillary should be collected in a 25 cc. volumetric flask. As it is almost impossible to stop the flow exactly on the mark, it is preferable to use a flask with a graduated neck. Another way of determining the volume is to stop the flow before it reaches the 25 cc. mark and then add water from a small buret to bring it to the mark; by subtracting the amount of water added, from 25 cc. the volume of flow is obtained. The same material should not be run through the plastometer a second time because the passage through the capillary tends to decrease the plasticity. The pressure causing the flow as read in centimeters of mercury should be changed to grams per square centimeter by taking into consideration the density of the mercury. The average hydrostatic fall in the plastometer should be determined as well as the density of the flour-water mixture; from these determinations the magnitude of the hydrostatic pressure in grams per square centimeter can be calculated. The actual pressure causing the flow is, therefore, the sum of the hydrostatic pressure and the air pressure expressed in grams per square centimeter.

Some of the authors here listed were undoubtedly working with plastic solids but they made no attempt to study them from that standpoint so their papers are included.

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XI. Protective Colloids

Expt. 47. Barium Sulfate Sol.—(*a*) Add to 5 or 10 cc. of a 0.5 per cent ammonium sulfate solution an equal volume of a 1 per cent barium chloride solution and mix thoroughly. Save for the purpose of comparison.

(*b*) Warm 25 cc. of the ammonium sulfate solution to about 60° C. and add to it 5 cc. of a warm 10 per cent pentosan gum sol such as U. S. P. gum acacia (gum arabic); mix thoroughly; and then add, with constant stirring, 25 cc. of the barium chloride solution heated to the same temperature. Fill a test tube with the mixture, allow to stand about 30 minutes and compare with the tube from (*a*). What effect has the presence of a protective colloid on the direct quantitative determination of the sulfate ion?

Expt. 48. Silver Chloride Sol.—Add carefully 0.1 *N* silver nitrate solution to 10 cc. of sodium protalbinate solution until a distinct precipitate of silver protalbinate has separated. Filter and wash it until the excess of silver nitrate has been removed. Then carefully bring the

precipitate into colloidal solution by the addition of 0.1 *N* sodium hydroxide solution. Examine this silver oxide sol by both reflected and transmitted light and record the colors. A portion of the solution can be dialyzed, if desired, to get a pure silver oxide sol. Add 10 per cent sodium chloride solution to the undialyzed silver oxide sol until a grayish white color appears. Dialyze the solution (Expt. 2) in the dark, until free from chlorides and then evaporate to dryness on the water bath. The residue when taken up with distilled water will again pass into colloidal solution if a drop of sodium hydroxide solution is added. What happens to the protalbinic acid when the solution is dialyzed?

Sodium protalbinate solution.—Suspend 5 gm. of protalbinic acid (Expt. 98) in 50 cc. of distilled water, add 0.1 *N* sodium hydroxide solution, and shake occasionally during the course of an hour. Again add sodium hydroxide and allow the mixture to stand over night when solution is practically complete. Then dilute to 100 cc. and filter. Preserve the solution with chloroform and toluene.

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Expt. 49. Determination of the Gold Number.—The gold number of an emulsoid colloid is defined by Zsigmondy as the number of milligrams of the emulsoid, which are just insufficient to prevent the change in color from red to violet in 10 cc. of a standard gold sol on the addition of 1 cc. of a 10 per cent sodium chloride solution. A gold sol having particles between 20 and 30$\mu\mu$ in diameter, is most suitable. The correct degree of subdivision may be known by a faint brownish opalescence in reflected light, and by a clear, deep red color in transmitted light. Prepare a 1 per cent solution of the substance whose gold number is to be determined. Place 10 cc. of the gold sol (Expt. 10) in each of four test tubes, (1), (2), (3), and (4). Add to the first three respectively 0.01, 0.1 and 1 cc. of the emulsoid sol, and mix thoroughly. After 3 to 5 minutes add to each of the four test tubes 1 cc. of a 10 per cent sodium chloride solution and again mix thoroughly. Five to ten minutes after the addition of the sodium chloride, compare the tubes. Assuming that there is a color change in (1) but not in (2) nor (3), the gold number must lie between 0.1 and 0.01. Tube (4) serves as a

check since it shows the effect of sodium chloride on the gold sol in the absence of a protective colloid. This should be preserved for purposes of comparison. For more accurate determinations 0.02, 0.05, and 0.07 cc., etc., of the protective colloid should be taken and the procedure repeated. If, however, this does not give results, dilute a portion of the original emulsoid and make determinations. The volume of the protective colloid used should never exceed 1 cc. Where the color change is not sharp, interpolation between unquestioned concentrations must be resorted to. Each student should determine the gold number of two substances, such as gelatin and U. S. P. gum acacia (gum arabic). To make the comparison of the gold numbers of different emulsoid sols clearer, each gold number may be expressed as the reciprocal of milligrams. The determination of the gold number serves as a delicate test for the characterization of proteins, and may be used to detect small amounts of a particular protein, when all other tests fail. This method has acquired importance in biochemical and medical work.

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Elliott, F. A., and Sheppard, S. E. The gold number of commercial gelatins. *J. Ind. Eng. Chem.*, 13, 699–700 (1921). A modification of Zsigmondy's method for the preparation of the gold sol is given.

Grey, F. T. Preparation of colloidal gold for the Lange test. *Biochem. J.*, 18, 448–450 (1924).

XII. Surface Tension, Surface Energy, and Adsorption

Expt. 50. Plateau's Experiment.—A large globule of oil placed on the surface of water immediately spreads out. If, however, a mixture of ethyl alcohol and water is prepared, with exactly the same specific gravity as the oil, and if the oil is introduced into the mixture with a pipet, it immediately takes the form of a sphere which remains suspended in the mixture. By suspending the oil in a liquid of the same specific gravity as the oil itself, it is removed from the influence of gravity and for this reason assumes the spherical form.

Place about 400 cc. of 60 per cent ethyl alcohol by volume in a 600–800 cc. beaker. Add from a pipet a few drops of refined cottonseed oil and notice whether they sink. If they do not sink add a small quantity of 70 per cent alcohol; stir and observe. If necessary add 50 per cent alcohol, or if the drops do not sink at all, add more 70 per cent alcohol. Continue the addition of the different concentrations of alcohol until a mixture, in which the drops sink about half way to the bottom, is obtained. Add more cottonseed oil by means of a pipet until a sphere an inch or more in diameter is obtained. Try changing the form by cutting the globule into two parts with a glass rod of small diameter. Does each part take a spherical form? Deform with a glass rod globules of different sizes. Which show most resistance to deformation, the large or small ones? Explain. Which most quickly regain their shape? It is observed that the glass rod is not wet by the oil when it comes in contact with it. Heat a polished copper wire in an alcohol flame until it develops an oxide film, and use it to deform the oil globules; the same result will be obtained as with the glass rod. Next heat another polished copper wire in a gas flame until a sulfide film is formed; apply it to the globules. The oil adheres to the wire and can be disengaged only with difficulty. The behavior of the glass rod and the copper sulfide wire towards the oil is similar to the siliceous gangue and mineral sulfides in the case of ore flotation.

*Plateau, J. Statique expérimentale et théorique des liquides soumis ausseules forces moléculaires. T. 1, pp. 53–55; t. 2, pp. 172–174. Gauthier-Villars, Paris, 1873.

Bancroft, W. D. The theory of emulsification. VI. *J. Phys. Chem.*, 19, 275–309 (1915).

Corliss, H. P., and Perkins, C. L. The theory of ore flotation. *J. Ind. Eng. Chem.*, 9, 481–488 (1917).

*Megraw, H. A. The flotation process. 2nd ed., p. 42. McGraw-Hill Book Company, New York, 1918.

Sulman, H. L. A contribution to the study of flotation. *Trans. Inst. Mining Met.*, 29, 44–204, 1920; published previously in *Bull. Inst. Mining Met.*, Nos. 182, 183 (1919), and 184 (1920).

Edser, E. The concentration of minerals by flotation. Fourth report on colloid chemistry and its general and industrial applications. *Brit. Assoc. Advancement Sci., Reports.* pp. 263–326, 1922.

Sulman, H. L. Note on an apparatus for small-scale flotation tests. *Bull. Inst. Mining Met.*, 220. pp. 1–10, 1923. Discussion on above article. *Bull. Inst. Mining Met.*, 221. pp. 1–8, 1923. Ten to twelve grams of ore may be treated in the apparatus described.

Expt. 51. Influence of the Degree of Curvature of the Surface Film on Internally Directed Pressure.—Blow a soap bubble on the end of a fire polished glass tube. Allow the bubble to contract spontane-

ously and observe how it collapses. Why does a curved film press inward? How many surfaces has a film? How may the pressure inside a soap bubble be calculated? To observe the effect of curvature, use the apparatus illustrated in Fig. 10. The tubes should have a diameter of about 1 cm., and the ends should be flaring. Close stop-cock *A* and blow a small soap bubble on one tube; close its stop-cock and repeat the process on the other tube, but make a larger bubble. Now open stop-cock *A*; observe what takes place and explain.

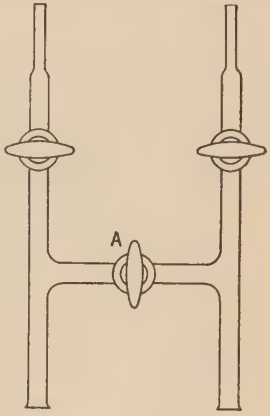

Fig. 10.

Soap solution.—Place 375 cc. of distilled water in a 500 cc. glass stoppered bottle; add 10 gm. of pure sodium oleate and allow the mixture to stand 24 hours to let the sodium oleate dissolve. Then add 125 cc. of pure glycerol, shake thoroughly, and allow the solution to stand in a dark place for a week. Siphon off the clear solution from the scum which will have collected on the surface. Add one or two drops of concentrated ammonium hydroxide to the solution, and preserve in a well-stoppered bottle in the dark. Do not use this stock solution every time a bubble is to be blown, but have a small working bottle. Never return any solution to the stock bottle, nor leave the stoppers out of the bottles nor allow the liquid to be exposed to the air more than is necessary. In preparing this solution *do not warm or filter it.*

Strutt, J. W. (Lord Rayleigh). Experiments upon surface-films. *Phil. Mag.* [5], 33, 363–373. Cf. 370 (1892); or Scientific papers. Vol. III, 1887–1892. pp. 562–571. Cambridge University Press, 1902.

Hendrick, F. B. Some lecture experiments on surface tension. *J. Phys. Chem.*, 16, 513–518 (1912).

*Boys, C. V. Soap-bubbles. Their colours and the forces which mould them. pp. 50–52 and 170–171. Society for Promoting Christian Knowledge, London, 1916.

Expt. 52. Changes in Surface Tension.—Thoroughly clean a large evaporating dish with chromic acid cleaning solution. Partially fill it with distilled water and place on the surface a small piece of camphor. Observe that the camphor at once begins a vigorous movement. This is due to the fact that the surface tension of the water is diminished as the camphor dissolves. Touch the surface of the water with the finger and observe the effect. The natural oil on the finger is transferred to the surface of the water as a film of grease, lowering its surface

tension; this usually arrests the movement of the camphor. The same result may be brought about more readily by touching the surface of the water with a glass rod to which a trace of pure olive oil adheres. Lord Rayleigh has calculated that the thickness of the film of oil necessary to stop the camphor movements lies between 1 and 2 $\mu\mu$. Some substances lower the surface tension of water, while other substances increase it. What effect have fatty acids, soaps, fats, and salts on the surface tension of water?

*Strutt, J. W. (Lord Rayleigh). Measurements of the amount of oil necessary in order to check the motions of camphor upon water. *Proc. Roy. Soc.* (*London*), 47, 364–367 (1890); or Scientific papers. Vol. III. 1887–1892. pp. 347–350. Cambridge University Press, 1902. The author considers that the motion of camphor upon the surface of water, long a puzzle, was satisfactorily explained by Van der Mensbrugghe in 1869.

Strutt, J. W. (Lord Rayleigh). Foam. *Proc. Roy. Inst. Gt. Brit.*, 13, 85–97. Cf. 89–91 (1890); or Scientific papers. Vol. III. 1887–1892. pp. 351–362. Cambridge University Press, 1902.

Strutt, J. W. (Lord Rayleigh). Experiments upon surface-films. *Phil. Mag.* [5], 33, 363–373. Cf. 366–368 (1892); or Scientific papers. Vol. III. 1887–1892. pp. 562–571. Cambridge University Press, 1902.

Expt. 53. Interfacial Tension.—Fill a Petri dish with distilled water to a depth of about 1 cm.; then using a pipet, place a globule of a mixture of chloroform and of an olive oil-oleic acid mixture (2 : 3) on the bottom of the dish under the water. Allow 0.1 N sodium hydroxide solution to diffuse against opposite sides of the drop at *exactly the same time and rate* by means of two pipets. Observe that the drop constricts and divides in two. This division is due to a decrease in surface tension at the poles, brought about by the formation of soap, and an increase of surface tension at the equator. Make a drawing of the process and explain it in detail. Try cutting a globule of the original mixture with a small cotton insulated wire, which has previously been soaked in 0.1 N sodium hydroxide. Repeat the experiment, using a wire soaked in 0.1 N acid solution. Explain the behavior of the alkali and acid. Cover a globule, which has had its surface tension lowered by previous treatment with alkali, with sufficient acid to neutralize the latter. Explain the results.

Olive oil-oleic acid mixture.—To 100 cc. of pure olive oil add 0.5 cc. of free oleic acid and mix thoroughly.

*McClendon, J. F. A note on the dynamics of cell division. *Arch. Entwickl. Organ.*, 34, 263–266 (1912).

Reynolds, W. C. On interfacial tension. Part II. The relation between interfacial and surface tension in sundry organic solvents in contact with aqueous solutions. *J. Chem. Soc.*, 119, 466–476 (1921).

Pound, J. R. Interfacial tension. *J. Chem. Soc.*, 123, 578–599 (1923).

Expt. 54. Effect of Electric Polarization.—Place about 10 cc. of clean mercury in a flat-bottomed vessel such as a Petri dish, and cover it with *N* potassium nitrate solution. Pass a direct current of eight or nine volts between the opposite sides of the globule; use either platinum or copper electrodes. The mercury becomes positively charged when placed in the potassium nitrate solution. The globule becomes less positive where the positive current enters; that is, the polarization decreases and the surface tension increases, while at the opposite pole the surface tension decreases. As the current increases, the pole at which the positive current enters, passes the neutral point and becomes negative, bringing about a decrease in surface tension. Thus the surface tension is decreased at one pole more than at the other. One pole is strongly positive and the other slightly negative, consequently the neutral region forms a band around the globule nearer to the negative pole than to the positive. This neutral band is the region of greatest surface tension. Where does the globule divide?

*Christiansen, von C. Kapillarelektrische Bewegungen. *Ann. Physik* [4], 12, 1072–1079. Cf. 1072–1074 (1903).

Freundlich, H. Kapillarchemie. Eine Darstellung der Chemie der Kolloide und verwandter Gebiete. Zweite Auflage, S. 412–413. Akademische Verlagsgesellschaft M. B. H., Leipzig, 1922.

Expt. 55. Effect of Adsorption of Ions on Surface Tension.—Place about 10 gm. of clean mercury in a small evaporating dish with a gently sloping bottom; cover it with dilute sulfuric acid (1 : 10), containing potassium dichromate sufficient to give the solution a light lemon-yellow color. When the mercury is covered with the mixture, its surface tension decreases and the globule flattens out. Touch the globule on one side with an iron nail; it contracts suddenly and breaks the contact, and then flattens out once more until it comes in contact with the nail, when it again contracts. A rhythmic pulsation of the mercury is thus produced. Lippmann found that when negative electricity was applied to mercury, its surface tension increased and the globule contracted. However, if sufficient negative electricity was added, the globule could be obtained in its original form; but when more was applied, it again expanded. The iron nail, when placed in the sulfuric acid solution, partially dissolves, going into solution as positively charged ions; this leaves the nail charged with negative electricity, which it imparts to the mercury when in contact with it. The potassium dichromate serves to oxidize the trace of mercurous sulfate formed on the surface. This is the principle applied in the construction of the Lippmann capillary electrometer.

Lippmann, G. Relations entre les phénomènes électriques et capillaires. *Ann. chim. phys.* [5], 5, 494–549 (1875).

Findlay, A. Practical physical chemistry. 4th ed., pp. 193–195. Longmans, Green and Company, New York, 1923.

Expt. 56. Adsorption of Dyes by Charcoal.—Place in each of two small Erlenmeyer flasks, respectively, 0.5 gm. of a vegetable decolorizing carbon, such as Norit. Add to one flask 25 cc., and to the other 100 cc. of methylene blue solution. Shake each flask, allow to stand half an hour, and then filter. Note the color of the filtrate in each case. Insert a test tube under the funnel used in filtering the contents of the first flask, and pour over the residue about 10 cc. of 95 per cent ethyl alcohol. Is the process reversible? How does the equilibrium concentration of the methylene blue in the organic solvent compare with that in water? What is the relationship of the surface tension of the two solvents? If a substance is to be decolorized with Norit, and it is equally soluble in water and alcohol, in which should it be dissolved and why?

Methylene blue solution.—Dissolve 0.5 gm. of methylene blue in 1000 cc. of distilled water.

Harkins, W. D., and Ewing, D. T. A high pressure due to adsorption, and the density and volume relations of charcoal. *J. Am. Chem. Soc.*, 43, 1787–1802 (1921). A paper on surface energy and surface forces.

*Bartell, F. E., and Miller, E. J. Adsorption by activated sugar charcoal. I. *J. Am. Chem. Soc.*, 44, 1866–1880 (1922).

Tanner, H. G. A lecture experiment demonstrating adsorption. *J. Am. Chem. Soc.*, 45, 437–438 (1923).

*Bartell, F. E., and Miller, E. J. Adsorption by activated sugar charcoal. II. *J. Am. Chem. Soc.*, 45, 1106–1115 (1923).

Miller, E. J. Adsorption by activated sugar charcoal. I. Proof of hydrolytic adsorption. *J. Am. Chem. Soc.*, 46, 1150–1158 (1924).

Bartell, F. E., and Miller, E. J. Adsorption by activated sugar charcoal. III. The mechanics of adsorption. *J. Phys. Chem.*, 28, 992–1000 (1924).

Expt. 57. Adsorption of Caramel by Charcoal.—Place in each of two Erlenmeyer flasks, respectively, 1 gm. and 2 gm. of either a vegetable decolorizing carbon, such as Norit, Carbrox, or Darco, or of bone charcoal. Add to each flask 100 cc. of the caramel solution, shake, allow to stand half an hour, and then filter. Explain the results. How is the principle, developed in this experiment, applied in the commercial clarification of sugars? All of the decolorizing carbons mentioned are most efficient, when the solution being decolorized has a hydrogen-ion concentration on the acid side. The caramel solution used has a hydrogen-ion concentration of about pH 3.5. The adsorption of hydrogen-ions by the charcoal causes it to become positively charged.

Caramel solution.—Place 12 gm. of sucrose in a small beaker, immerse this is in a sulfuric acid bath, and heat at 212° C. for half an hour, stirring constantly. The sugar melts before caramelizing. Dissolve the caramel in 200 cc. of boiling distilled water and dilute to 1750 cc.

*Wiley, H. W. Experiments in the culture of sugar cane and its manufacture into table sirup. *U. S. Dept. Agr., Bur. Chem. Bull.*, 93. p. 65, 1905. The preparation of a caramel color standard is described.

Bock, J. C. A study of a decolorizing carbon. *J. Am. Chem. Soc.*, 42, 1564–1569 (1920). Some adsorptive properties of Norit are described.

*Tanner, H. G. Potash from kelp. VI—The decolorizing of adsorptive charcoals. *J. Ind. Eng. Chem.*, 14, 441–443 (1922).

Bartell, F. E., and Miller, E. J. Adsorption by activated sugar charcoal. I. *J. Am. Chem. Soc.*, 44, 1866–1880 (1922).

Bartell, F. E., and Miller, E. J. Adsorption by activated sugar charcoal. II. *J. Am. Chem. Soc.*, 45, 1106–1115 (1923).

Simpson, S. G. Formation of formic acid by caramelization of cane sugar. *Ind. Eng. Chem.*, 15, 1054–1055 (1923).

Expt. 58. Adsorption of Protein by Filter-Cel.—Place 20 cc. of a 1 per cent egg albumin solution in a large test tube or small Erlenmeyer flask; add 4 gm. of a *filtering aid* such as Filter-Cel; shake vigorously for about two minutes, and filter. Test the original solution and the filtrate for protein by both the biuret (Expt. 76) and Millon solutions (Expt. 78). The filtering aid produces a porous cake, making possible a rapid filtration of solutions which otherwise are filtered with difficulty; it also adsorbs some of the substances in the solution.

*Derleth, C. P. Filter aids. *J. Ind. Eng. Chem.*, 13, 989–990 (1921).

Hickey, G. M. The use of Filter-Cel for industrial filtration processes. *J. Ind. Eng. Chem.*, 13, 990–992 (1921).

Caldwell, J. S. Studies in the clarification of unfermented fruit juices. *U. S. Dept. Agr., Prof. Paper, Bull.*, 1025, 1922.

Expt. 59. Adsorption of Alkaloids by Lloyd's Alkaloidal Reagent.—Aklaloids and their salts are adsorbed from neutral or acid water solutions by Lloyd's alkaloidal reagent (colloidal hydrous aluminum silicate). Dissolve 0.3 gm. of quinine bisulfate in 25 cc. of distilled water and taste. Slightly acidify 5 cc. of the solution with dilute hydrochloric or sulfuric acid and test for the presence of an alkaloid with Mayer's reagent. To the remainder of the quinine solution add 4 gm. of Lloyd's reagent, shake vigorously, and filter. The reaction is nearly instantaneous. Taste the filtrate and repeat the test with Mayer's reagent. Taste the residue. Shake the residue with 10 cc. of 0.1 *N* sodium hydroxide solution, filter, and neutralize the filtrate with 10 cc. of 0.1 *N* hydrochloric or sulfuric acid. Test a small portion with Mayer's

reagent. Explain the results. How does the change of reaction affect adsorption by Lloyd's reagent? What effect has the specific surface of a substance on adsorption?

Mayer's reagent (potassium mercuric iodide solution).—Dissolve 3.4 gm. of mercuric chloride and 12.5 gm. of potassium iodide separately in water, mix the solutions, and dilute to 500 cc. This solution gives a test for minute traces of alkaloids in solutions slightly acidified with either hydrochloric or sulfuric acids.

*Lloyd, J. U. Process of extracting, purifying, or excluding alkaloids and alkaloidal salts. U. S. Patent 1,048,712. Dated Dec. 31, 1912.

Lloyd, J. U. Alkaloidal substance. U. S. Patent 1,048,711. Dated Dec. 31, 1912.

*Lloyd, J. U. Discovery of the alkaloidal affinities of hydrous aluminum silicate. *J. Am. Pharm. Assoc.*, 5, 381–390; 490–495 (1916).

Folin, O., and Berglund, H. A. A colorimetric method for the determination of sugars in normal human urine. *J. Biol. Chem.*, 51, 209–211 (1922). The method depends upon the fact that Lloyd's alkaloidal reagent removes most of the coloring matter, the uric acid, the creatine, and the creatinine, and yet does not affect the sugar.

Expt. 60. Capillary Analysis.—The ascent of colloids to different heights on strips of filter paper is believed by Fichter and Sahlbom to be due to the difference in the electric charge carried by the colloid. Negatively charged colloids rise with their dispersion media, while positively charged colloids are held near the surface of the liquid. Place 25–50 cc. of each of two basic dyes, such as methylene blue, malachite green, crystal violet, and safranin, and the same amount of each of two acid dyes, such as acid fuchsine, and ponceau, in small beakers. In each, suspend a strip of filter paper, 25 × 2 cm., from a horizontal glass rod supported by a clamp; allow 2–3 cm. of the paper to dip into the dye solution. At the end of an hour, observe the height in millimeters to which the dyes have risen. What electrical charge does filter paper assume when in contact with water? Account for the difference in the ascent of the dyes.

Dye solutions.—Dissolve 0.05 gm. of a dye, such as methylene blue, malachite green, crystal violet, safranin, acid fuchsine and ponceau, in 500 cc. of distilled water.

Goppelsroeder, F. Neue Capillar- und Capillaranalytische Untersuchungen. *Verh. Naturf. Ges., Basel*, 19, Heft 2, 1–81. Mit 52 Tafeln Textbeleg (1907).

*Pelet-Jolivet, L., und Jess, C. Ueber den kapillaren Aufstieg von Farblösungen. *Kolloid-Z.*, 3, 275–280 (1908).

Pelet-Jolivet, L., Ueber den kapillaren Aufstieg der Farbstoffe. *Kolloid-Z.*, 5, 238–243 (1909).

Goppelsroeder, F. Ueber Kapillar- und Adsorptionsanalyse. *Kolloid-Z.*, 4, 23–27, 94–123, 191–197, 236–252, 312–315 (1909); 5, 52–62, 109–116, 159–169,

200–211, 250–260, 305–317 (1909); 6, 42–50, 111–122, 174–179, 213–221, 268–274 (1910). A review of the work done between 1861 and 1907.

Fichter, F., und Sahlbom, N. Die Kapillaranalyse kolloidaler Lösungen. *Verh. Naturf. Ges., Basel*, 21, 1–24. 18 Figur (1910).

Freundlich, H. Grundzüge der Kolloidlehre. S. 50–52. Akademische Verlagsgesellschaft M. B. H., Leipzig, 1924.

Expt. 61. Determination of an Adsorption Isotherm.—Prepare 250 cc. of an $M/3$ (0.65 N) solution of crystallized oxalic acid. Place in each of five small Erlenmeyer flasks the following: 50 cc. of the original oxalic acid solution; 40 cc. of solution and 10 cc. of distilled water; 30 cc. of solution and 20 cc. of distilled water; 20 cc. of solution and 30 cc. of distilled water, and 10 cc. of solution and 40 cc. of distilled water. Number the flasks in the same order, 5, 4, 3, 2, and 1 respectively, these being the ratios of their original concentrations. Place in each flask 1 gm. of finely powdered vegetable decolorizing carbon, such as Norit; shake thoroughly at frequent intervals for 30 minutes and then clarify by centrifuging or by allowing the charcoal to settle over night.

The potassium permanganate solution should be of such strength that 5 cc. will equal 1 cc. of the original oxalic acid solution. Prepare 500 cc. of an $M/15$ (0.13 N) solution of potassium permanganate. The actual ratio of permanganate solution to oxalic acid should be determined. Place 5 cc. of the stock oxalic acid solution in a 400 cc. beaker, add 200–250 cc. of hot water at 80–90° C., and 10 cc. of sulfuric acid (1 : 1). Titrate at once with the permanganate solution, stirring vigorously and continuously. The last cubic centimeter must be added drop by drop with particular care to allow each to be fully decolorized before the next is introduced. The excess of potassium permanganate required to cause an end point must be determined by matching the color in another beaker containing the same amount of water and acid. The solution should not be below 60° C. by the time the end point is reached. Rapid cooling may be prevented by allowing the beaker to stand on a hot plate during the titration. The use of a thermometer as a stirring rod is convenient in these titrations.

Pipet 5 cc. of the solution from each of the five flasks without disturbing the sediment of charcoal, and titrate each. Make duplicate determinations in each case. Begin with (5) and either back titrate or repeat the determination if the end point is overrun. From the calculated amount of permanganate solution required by 5 cc. of the different oxalic acid solutions before adsorption, deduct the cubic centimeters of permanganate solution required after adsorption. The difference gives the amount of oxalic acid adsorbed, expressed in cubic centimeters of standard potassium permanganate.

While no adsorption formula, thus far advanced, exactly conforms to the experimental data over an extended range, the simplest and most acceptable is that by Freundlich, as follows:

$$\frac{x}{m} = aC^{1/n},$$

in which x = the amount of substance adsorbed,
m = the weight of adsorbing substance,
C = the concentration of the substance in solution at equilibrium,
a and $1/n$ are constants.

These constants depend upon the nature of the substances used, and upon the solvent.

In the determinations carried out above, x is the number of cubic centimeters of potassium permanganate solution equivalent to the oxalic acid which was adsorbed, and C is the number of cubic centimeters of potassium permanganate solution equivalent to the oxalic acid remaining in the solution at equilibrium. Plot the values of x/m as ordinates and C as abscissae on coordinate paper using a convenient scale, such as 1 cc. = 1 cm. For true adsorption the points should lie on a smooth curve of the parabolic type.

The parabolic curve obtained may be smooth and have the appearance of an adsorption isotherm, yet it is impossible to say definitely that it conforms to the equation without a further test. Whenever data seem to exhibit a uniform *rate* of increase or whenever it is desired to study the *relative* changes rather than the *actual* changes in data, a *logarithmic curve* should be used. Such a curve is obtained by taking the logarithms of the measurements and using these logarithms as coordinates. Since multiplying two numbers adds their logarithms, a constant rate or ratio will appear in the logarithmic diagram as a constant addition. Hence if there is a constant rate in the reaction the logarithmic curve will be a straight line. A test of the validity of the adsorption equation is to take the logarithms on both sides of the equation:

$$\log x = 1/n \log C + \log a.$$

Using $\log x$ and $\log C$ as coordinates this is the equation for a *straight line.* Plot the logarithms of x as ordinates and those of C as abscissae, to a convenient scale, such as 0.1 = 1 cm. on ordinary coordinate paper. The logarithms should be read to three figures. The same curve can also be obtained by plotting the actual figures direct on logarithmically ruled paper.

The log x − log C curve may be used for determining the value of n in the equation of the isotherm; since n = log C/ (log x − log a) is the cotangent of the angle made by the straight line with the C-axis. Calculate the value of n in the present case, and from this the value of $1/n$. Log a is the value assumed by log x when log C = 0. This is the length cut off by the straight line on the x-axis. What is the value of a log a in the diagram?

Both the adsorption isotherm and the log x-log C curve may be plotted with arbitrary units as coordinates, for any given solute; however, comparison with another substance can be made only by expressing the results as molar concentrations. Calculate the molar concentration of the original oxalic acid solution in contact with 1 gm. of charcoal in each of the five flasks. Again calculate the molar concentration of the solution after adsorption has taken place in each flask. These results permit comparison of oxalic acid with another organic acid, using the same quantities of charcoal and concentrations of acid.

Walker, J., and Appleyard, J. R. Absorption of dilute acids by silk. *J. Chem. Soc.*, 69, 1334–1349 (1896).

Freundlich, H. Kapillarchemie. S. 145–173. Leipzig. 1909.

McBride, R. S. Standardization of potassium permanganate solution by sodium oxalate. *Bur. Standards. Sci. Tech. Papers* No. 182. p. 32. 1913; also *J. Am. Chem. Soc.*, 34, 393–416 (1912).

*Willows, R. S., and Hatschek, E. Surface tension and surface energy and their influence on chemical phenomena. pp. 63–68. P. Blakiston's Son & Co., Philadelphia, 1919.

*Hatschek, E. Laboratory manual of elementary colloid chemistry. pp. 116–123. J. & A. Churchill, London, 1920. The experiment is largely taken from this excellent description.

Bock, J. C. A study of decolorizing carbon. *J. Am. Chem. Soc.*, 42, 1564–1569 (1920).

Expt. 62. Adsorption of Dye at Liquid-gas Interface.—Number consecutively three test tubes and fill the first about two-thirds full of either aniline green or aniline blue solution. Close this test tube with a two-hole rubber stopper. This carries an inlet tube, which extends almost to the bottom of the test tube and has the lower end drawn out to a capillary, and a delivery tube, which is inserted in the second test tube, as shown in Fig. 11. Connect the inlet tube with a compressed air tank or a cylinder of an inert gas, such as carbon dioxide or hydrogen, and adjust the pressure and position of the tube in the solution so that froth will be formed and forced through the delivery tube into the second

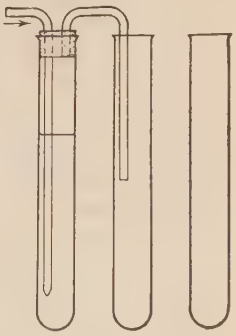

Fig. 11.

test tube. Care must be taken to make sure that froth and not liquid is forced over. Continue the pressure until two-thirds of the solution has bubbled over. Now transfer the stopper containing the tubes from the first to the second test tube and bubble half of its contents into the third test tube. Allow the froth in each of the three tubes to break and compare each resulting solution with the original dye solution as a standard.

Make color comparisons with a colorimeter, such as the Duboscq or Kober. By the use of this instrument the intensity of color in the two solutions can be accurately measured; and also calculations of the comparative amounts of substances, which quantitatively form these colored compounds, may be made. The depth of the colored solutions through which the light passes is regulated by raising or lowering the cups; the scale on the instrument accurately indicates this in millimeters. Place in one of the cups a small amount of the standard solution and in the other, the unknown solution. Adjust the standard solution to a convenient depth, preferably to an exact number of millimeters, and match with it the color intensity of the unknown solution by raising or lowering the latter. Record the readings of the scale and vernier. For purposes of accuracy repeat the operation several times and take the average of the readings. The amounts of the colored substance in the solutions are inversely proportional to the depths of the columns of liquids.

Thus, if C_1 = the concentration of the standard solution; D_1 = the depth of the standard solution; C_2 = the concentration of the unknown solution; and D_2 = the depth of the unknown solution, then

$$C_2 : C_1 :: D_1 : D_2 \quad \text{and} \quad C_2 = \frac{C_1 \times D_1}{D_2}.$$

After making the color comparisons, rinse tube (1) with distilled water until all the visible dye is removed; then add 10 cc. of 95 per cent ethyl alcohol and shake. Explain the change that takes place.

Dye solutions.—Dissolve 0.05 gm. of a dye such as aniline green or aniline blue in 250 cc. of distilled water.

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Expt. 63. Coagulation of Saponin at Liquid-gas Interface.—Using a 5–10 per cent saponin solution, blow a bubble 3–4 inches in diameter on the end of a fire-polished glass tube. Hold the bubble for several seconds and then slowly suck the air out or allow it to contract spontaneously. Compare the collapse of a saponin bubble with that of a soap bubble (Expt. 51). In the case of saponin, the film has ceased to be elastic and can collapse only by falling into folds. It should be possible to see little rods of the solid in the film. This concentration of saponin in the surface film results in coagulation. Egg albumin and peptone behave in a similar manner. What have investigations shown in regard to the thickness of surface films?

*RAMSDEN, W. Separation of solids in surface-layers of solutions and suspensions. *Proc. Roy. Soc.* (*London*), 72, 156–164. Cf. 158 (1904).

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Expt. 64. Adsorption as Preliminary to Chemical Reaction.—Bayliss points out that when a reaction occurs in a heterogeneous system certain preliminary processes take place. Diffusion is the first stage, adsorption the second, and chemical reaction the third and last. The latter is, as a rule, the stage which conditions the rate of the process as a whole. The substance adsorbed may be either in true solution or colloidal solution. If the adsorbed substance does not enter into chemical combination with the substance upon whose surface it is adsorbed, it nevertheless forms a kind of complex, which may be separated from the rest of the system. These substances have been called "adsorption compounds."

A colloidal solution of the free acid of Congo red is blue, while the salt is red. To 10 cc. of alumina cream add 4 cc. of the free acid of Congo red and 50 cc. of distilled water. Shake thoroughly, centrifuge about two minutes, and at once decant the supernatant liquid. What is the color of the adsorption compound? In it, acid and base exist side by side but uncombined. Suspend it immediately in 10–15 cc. of distilled water and allow to stand at room temperature. On standing, chemical combination of acid and base occurs with the formation of the aluminum salt of Congo red. The adsorption compound seems to be the result of the mutual precipitation of the electropositive and electronegative colloids, aluminum hydroxide and Congo red acid.

Congo red solution.—Dissolve 0.5 gm. of Congo red (sodium salt of diphenyl-diazo-binaphthionic acid) in 100 cc. of distilled water.

Free acid of Congo red.—To 100 cc. of Congo red solution add 9 cc. of 0.1 *N* hydrochloric acid. If an excess of acid should be added, the free acid is precipitated, and this on dialysis gives a deep blue colloidal solution.

Aluminum cream.—To an alum or aluminum sulfate solution, add ammonium hydroxide with constant stirring, until the solution is slightly alkaline to litmus. Wash the precipitate by decantation with distilled water, until the wash water shows only a trace of sulfate when tested with barium chloride. Decant the excess of water and preserve the resulting suspension of aluminum hydroxide.

*Bayliss, W. M. The properties of colloidal systems. II. On adsorption as preliminary to chemical reaction. *Proc. Roy. Soc.* (*London*) [B], 84, 81–98. Cf. 83 (1911–12).

*Polarimetry. *Bur. Standards, Circ.* 44. pp. 64–65, 1918.

Expt. 65. Adsorption as Preliminary to Enzyme Action.—Place in each of two small Erlenmeyer flasks, (1) and (2), 1 gm. of granular fibrin and 50 cc. of 0.2 per cent hydrochloric acid; allow both to stand for an hour. Add 5 cc. of 1 per cent pepsin solution to flask (1), shake thoroughly, filter, and collect the filtrate in flask (3). To this add 0.5 gm. of fresh fibrin. Place 50 cc. of fresh hydrochloric acid in flask (4) and add to it the residue from flask (1). Next add 5 cc. of 1 per cent pepsin solution to flask (2). Incubate the three mixtures at 37° C.; observe at intervals during the first few hours and again at the end of 24 hours. Explain the results.

von Wittich. Das Pepsin und seine Wirkung auf Blutfibrin. *Arch. ges. Physiol.* (*Pflüger's*), 5, 435–469. Cf. 444 (1872).

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CHAPTER II

PHYSICAL CHEMICAL CONSTANTS OF PLANT SAPS

Expt. 66. Representative Sample of a Plant Sap.—When making a study of the properties of plant saps, it is essential to secure a representative sample. Dixon and Atkins have observed that samples of plant sap, extracted successively from unfrozen tissue can not be regarded as typical, because they differ in the depression of the freezing point and electrical conductivity. Therefore, to obtain a representative sample it is necessary to render the cell membranes permeable by freezing the tissue before the extraction of the sap. For accomplishing this they recommend immersion in liquid air, but Gortner and Harris use a mixture of ice and common salt.

Place the plant tissue [1] in a rubber-stoppered bottle and pack it in a slushy mixture of pulverized ice and common salt or calcium chloride, contained in an earthenware jar, which fits snugly inside a well-insulated "fireless cooker." A temperature of -15 to $-17°$ C. can be obtained easily with ice and common salt, while the use of calcium chloride produces a much lower temperature, which is desirable in many cases. After the tissue has been frozen for at least 8 hours, thaw it by placing the bottle under running tap water, rinse with distilled water and wipe dry before opening. Fit 8 oz. cotton duck into a small steel cup and place in it the thawed tissue. Express the plant sap in either a hydraulic press or a hand-screw press, which permits the application of heavy pressure. Keep the parts of the press which come in contact with the sap coated with a thin layer of paraffin. Centrifuge the tissue fluid to remove any suspended cell debris.

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*Gortner, R. A., and Harris, J. A. Notes on the technique of the determination of the depression of the freezing point of vegetable saps. *Plant World*, 17, 49–53 (1914).

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[1] Cabbage (*Brassica oleracea*) may be used in this and Expts. 67, 68, 69, and 70.

Expt. 67. Moisture Content of a Plant Sap.—Sugar investigators have found that the refractive index of sugar solutions gives them a reliable indication of the total quantity of solids present. For determining this they use an Abbé refractometer, which, in addition to the refractive index scale, has a special sugar scale from which the percentage of sugar in a sirup may be read directly. Gortner and Hoffman applied this principle to the determination of the moisture content of a plant sap. They found that the refractive indices of solutions of inorganic salts and proteins in the *concentrations normally present in plant saps* gave the same readings on the refractometer as the corresponding sugar solutions.

The essential parts of an Abbé refractometer (Fig. 12) are a compensator and two flint-glass prisms, each having a refractive index $n_D = 1.75$, and each being cemented into a metal mounting, the one hinged, the other fixed, but both so attached to an alidade that they can be rotated on a horizontal axis. In addition, the instrument has a telescope, fastened to a sector on which the refractive index and the sugar scale are engraved. The compensator is attached to the lower end of the telescope. To charge the refractometer, first clamp the alidade into a position parallel with the telescope, then rotate the instrument on its bearings to a horizontal position so that the movable prism is uppermost; release the screw head holding the two prisms together and swing open the movable prism. Place a few drops of the liquid, whose index of refraction is to be measured, upon the polished surface of the fixed prism. Close and fasten the prisms firmly by tightening the screw head. Then swing the instrument into an upright position. Since the refractive index of liquids varies with the temperature, maintain the double prisms at a constant temperature by circulating through them water at 20° C. Allow both instrument and sample to reach the same temperature before the reading is made. Use either an electric lamp or a Welsbach burner as a source of light, although ordinary daylight will serve in many cases.

To set the instrument for observation bring the *border line* into the field of the telescope, rotating the prisms by means of the alidade in the following manner: Hold the sector firmly, and move the alidade from its initial position, at which the refractive index points $n_D = 1.3$, in the ascending scale until the intersection of the reticule in the telescope cuts the dividing line between the bright and dark portions of the field. This line of division is called the *border line.*

To obtain practice in the use of the Abbé refractometer, determine the refractive indices of pure olive oil, pure cottonseed oil, and a mixture of the two oils prepared by adding equal volumes from a pipet and

mixing thoroughly. Take five concordant readings using the average in each case.

Plot a graph on coordinate paper using the refractive index readings as the ordinates and percentage composition as the abscissae. Since the refractive index of each of the pure oils and of the mixture is known,

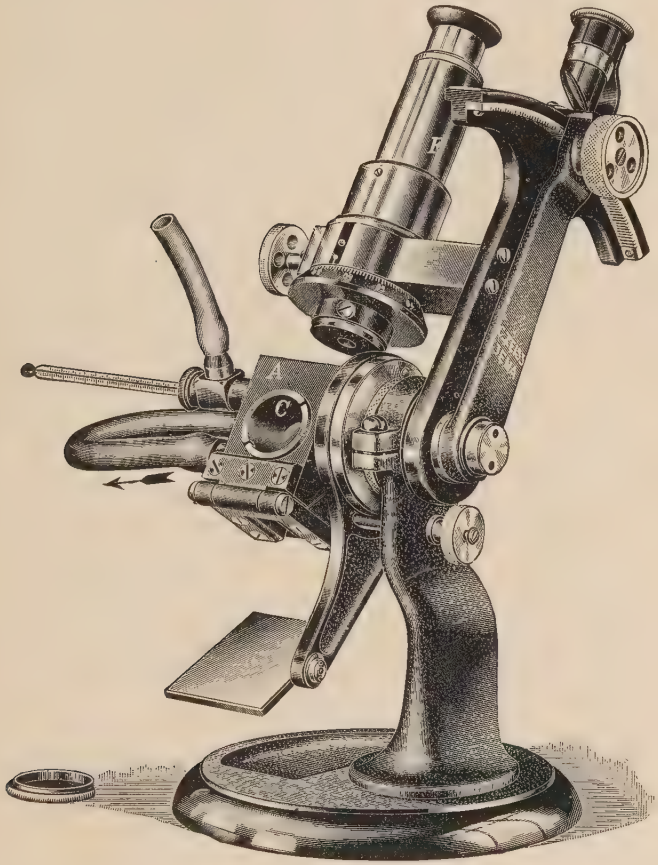

Fig. 12.

it is possible to determine the percentage composition of the two components.

To determine the moisture content of a plant sap, place two or three drops of expressed plant sap (Expt. 66) or of a sap freshly drawn from a plant upon the polished surface of one of the prisms of an Abbé refractometer and adjust it to position. As soon as the temperature has reached 20° C. read the percentage of total solids. To determine the

percentage moisture content, subtract from 100 the percentage of total solids. After the readings have been taken the prisms should be cleaned with ethyl alcohol and cotton.

The accuracy of the adjustment of the instrument should be tested from time to time. This may be done by using distilled water and taking the average of several readings. The refractive index of water at 20° C. is 1.3330, expressed as n^{20}.

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Expt. 68. Osmotic Pressure of a Plant Sap.—The concentration of a gas can be calculated, providing the temperature, the volume, and the pressure are known. Thus, Boyle's law states that *the volume of a given mass of gas kept at constant temperature varies inversely with the pressure.* Charles' law states that *the volume of a given mass of gas kept under constant pressure varies directly as the absolute temperature.* Combining these with Avogadro's law that *equal volumes of gases under like conditions of temperature and pressure contain the same number of molecules* gives the formula:

$$PV = nRT \qquad (1)^2$$

where P = Pressure
V = Volume
n = the number of mols of gas in volume V
R = the molecular gas constant
T = absolute temperature

From this formula, it is possible to calculate the pressure when the other quantities are known:

$$P = \frac{nRT}{V}. \qquad (2)^2$$

Actual measurements show that one gram molecule of a gas occupies a volume of approximately 22.4 liters at 0° C. and one atmosphere pressure; conversely therefore one gram molecule, when contained in a volume of 1 liter at 0° C., would exhibit a pressure of 22.4 atmospheres.

Soon after the demonstrations of the gas laws it was found that *solutions approximately obey the same laws providing that V is the volume*

[2] Since these formulae apply to an *ideal* gas, certain corrections must be made for exact physico-chemical work with actual gases.

of the solvent. Theoretically therefore it follows that one gram molecule of a non-electrolyte dissolved in 1 liter of solvent exhibits a pressure of approximately 22.4 atmospheres. This pressure is known as the *osmotic pressure* of the solution.

Various methods have been devised to measure osmotic pressure.

(*a*) The *direct method* involves the separation of the solution from the pure solvent by a membrane freely permeable to the molecules of the solvent, but impermeable to the molecules of the solute. If the solution is in a closed container connected with an instrument capable of recording pressures, it will be found that the solvent diffuses into the solution and that *at equilibrium* a pressure of approximately 22.4 atmospheres is recorded, providing that the equilibrium solution contains one mol of solute in one liter of solvent. If the solute is a highly ionized electrolyte, each ion will contribute toward the osmotic pressure so that if the solute is completely ionized into two ions, a pressure of approximately 44.8 atmospheres would be observed. The technical difficulties of making direct measurements are such that certain indirect methods are more commonly employed.

Such indirect measurements may be (*b*) plasmolytic methods, (*c*) vapor pressure measurements, (*d*) the measurement of the elevation of the boiling point, or (*e*) the depression of the freezing point of the solution.

(*b*) In the *plasmolytic methods,* a section of the plant or animal tissue containing uninjured cells is placed in a series of solutions of known but varying concentrations, and the cells are observed under the microscope to find the concentration of the external solution which just causes the protoplasm to contract and draw away from the cell membrane. This contraction is due to the withdrawal of water from the protoplasm to the more concentrated external solution. The solution of equal osmotic pressure to the solution in the cell is slightly more dilute than is the solution which will cause visible plasmolysis.

(*c*) *Vapor pressure methods* depend upon the fact that a solution has a lower vapor pressure than has the pure solvent. Consequently the vapor of the solvent will distill from the pure solvent through the pure vapor of a solution.

(*d*) *Elevation of the boiling point* is a natural corollary of the decrease in vapor pressure of a solution. Unfortunately this method is of but little importance in biochemistry because so many of the biochemical systems are altered by heat.

(*e*) *Depression of the freezing point* is the method usually employed in biochemical studies and it has been extensively applied to studies of plant saps.

Cryoscopic method.—The essential apparatus includes:

(1) A Beckmann thermometer, T, graduated in $\frac{1}{100}$ or preferably, since water is the solvent, a Heidenhain thermometer, reading from 1° to − 5° C., graduated in $\frac{1}{100}$ intervals.

(2) A glass tube, A, serving as a container for the plant sap and fitted with a stopper having openings for the thermometer and stirring device.

(3) A stirrer, S, in the form of a coil of platinum or nickel wire coiled around the thermometer bulb and extending up through the stopper, so that it can be easily moved up and down during the actual freezing process.

(4) A freezing bath, B, of ice and salt, the temperature of which is approximately − 6 to − 8° C. Through the cover of the freezing bath, there is inserted a tube, A_1, larger than the one containing the sap. This larger tube serves as a cold air thermostat to prevent actual physical contact of the tube, containing the sap, with the ice and salt mixture. A diagram of the apparatus as assembled is shown in Fig. 13.

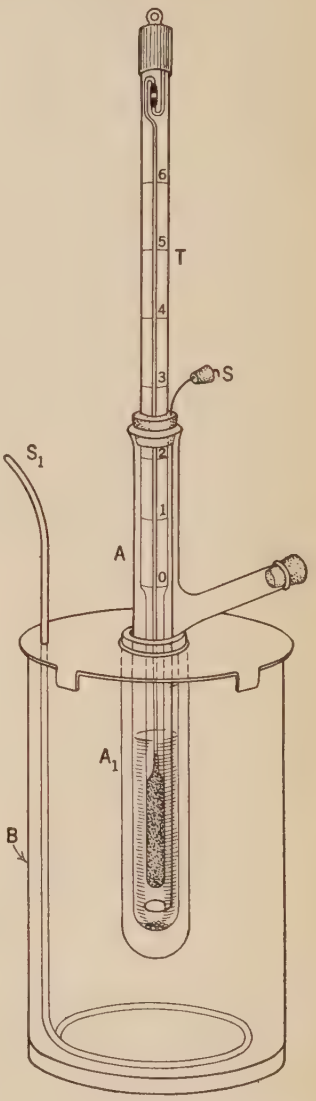

Fig. 13.

Place a portion of a sap (Expt. 66) in the freezing point tube. Close with a stopper containing the stirrer and thermometer and insert all in the cold air thermostat. Stir constantly and observe that the mercury column slowly recedes in the thermometer to a point considerably below the true freezing point. Note approximately the lowest temperature reached by the mercury thread. When actual freezing begins there is a rebound of the mercury. The heat caused by the crystallization of ice

raises the temperature, and the mercury thread races up the capillary until it finally reaches a maximum value which is maintained for a half minute or more. *This is the observed freezing point.* Determine the true zero point on the thermometer by freezing distilled water in place of the plant sap; disregard the under cooling, since it does not affect the freezing point. Determine the observed depression of the freezing point, Δ', by calculating the difference in degrees Centigrade between the observed freezing point of the sap and the true zero point on the thermometer. Upon this basis calculate the corrected depression of the freezing point, Δ, by the following formula:

$$\Delta = \Delta' - 0.0125U\Delta',$$

where U = degrees of under-cooling below the observed freezing point.

This formula is based on the fact that for each degree of under-cooling of water, .0125 or $\frac{1}{80}$ of the water freezes, and the heat of crystallization of this ice is sufficient to raise the temperature of the system to 0° C. This crystallization of the ice causes the solution to become more concentrated so that the observed freezing point is the freezing point of a solution more concentrated than was the original sap. The corrected freezing point approximates the freezing point of the original sap, assuming that crystallization began at the instant the true freezing point was reached and that only an insignificant amount of water was transformed into ice. If the freezing point of the sap is known approximately, it is possible to add a flake of hoar frost to start crystallization and thus obviate the necessity of correcting for the under-cooling.

An example of the above calculations is as follows:

(1) Pure water froze at. . — .200° C.
(2) Sap cooled to. . . . . — 4.600° C.
(3) Sap froze at. . . . . — 2.300° C.

U = (2) − (3) = 2.300° C.
Δ' = (3) − (1) = 2.100° C.
Δ = 2.100 − (.0125 × 2.300 × 2.100)
Δ = 2.100 − 0.050
Δ = 2.050° C.

This depression of the freezing point may be approximately converted into osmotic pressure (P) by the formula:

$$P = 12.06\Delta - 0.021\Delta^2$$

The osmotic pressure of a plant sap is not a measure of the molecules of dissolved materials alone but the ions likewise contribute to the

observed values. The values which are obtained are for an equilibrium at the temperature at which the sap freezes and may differ appreciably from the values in the actively growing plant tissue.

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Expt. 69. Average Molecular Weight of Solutes in a Plant Sap.—The average molecular weight of the dissolved solutes in a plant sap is calculated from (1) the determination of the total solids of the plant sap (Expt. 67), and (2) the measurement of the depression of the freezing point (Expt. 68). That is

$$M = C \cdot \frac{K}{\Delta}$$

where M = the average molecular weight;

C = the concentration of the solutes, $\frac{\text{weight of solutes}}{\text{weight of solvent}}$;

K = 1000 × the molecular lowering for a given solvent, and

Δ = the depression of the freezing point in degrees centigrade.

In order to facilitate the calculations, Harris and Gortner have published tables for $\frac{K}{\Delta}$, using 1.86° as the depression of the freezing point produced by dissolving one mol of solute in 1000 gm. of water. The solutes comprise both organic and inorganic substances, such as sugars and salts, and it is reasonable to suppose that the former have a greater molecular weight than the average of the ions and undissociated molecules of the latter. To show changes in relative concentration of ions in relation to total solutes, Harris and Gortner used the value, $\frac{\kappa}{\Delta}$, κ being the specific electrical conductivity of the sap.

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Expt. 70. Hydrophylic Colloid Content of a Plant Sap.—The emulsoid character and the water binding power of the colloids, present in living material, are rapidly being recognized as most important. Newton and Gortner have developed a method whereby a *relative* value for the "bound" water in an expressed plant sap may be estimated. The freezing point depression of the sap is first determined. Then, having found the total solids by the refractometer method, there should be added to a fresh portion of the plant sap, a quantity of sucrose just sufficient to make a molar solution in the total quantity of water present. The freezing point depression of this solution should be determined. According to evidence from Scatchard, the sucrose forms a hexahydrate in solution, and the theoretical depression is thus 2.085° C. The actual depression is usually found to be greater than this, because the hydrophilic colloids bind some of the water and thus prevent it from functioning as a solvent for the sucrose. The sucrose solution is therefore more concentrated than is indicated by the calculated value and consequently it freezes at a lower temperature. It seems probable that the relative content of hydrophylic colloids in other biological fluids may be determined by this method.

For the experiment, use winter hardened plants, such as wheat (*Triticum vulgare*), or some xerophytic plant, as the cactus (*Opuntia* spp.). Express the plant sap (Expt. 66). Determine the depression of its freezing point, using the standard Beckmann apparatus (Expt. 68). Then having found the total solids by the refractometer (Expt. 67), weigh out a fresh portion of the sap sufficient to contain 10.0 gm. of water. Place in a dry Beckmann freezing-point tube and cool to about 0° C. Add to this 3.4224 gm. of finely pulverized sucrose, and keep the mixture at a low temperature to prevent any invertase present from hydrolyzing the sucrose during solution. The quantity of sucrose used is just sufficient to make a molar solution in the 10 gm. of water. Determine the freezing point of the mixture.

TABLE III

Materials Used	Total Solids, Per Cent	Δ	Δ_a	$\Delta_a - \Delta$	$\Delta_a - (\Delta + K_m)$	Bound Water, Per Cent
Hardy winter wheat						
Triticum vulgare var. Buffum	17.8	1.719	4.158	2.439	0.354	13.0
Semi-hardy winter wheat						
Triticum vulgare var. Turkey	13.5	1.273	3.612	2.339	0.254	9.7
Non-hardy winter wheat						
Triticum vulgare var. Super	9.7	1.085	3.279	2.194	0.109	4.4
Greenhouse plant						
Cereus	4.9	0.505	2.803	2.298	0.213	8.3
Solutions of gum acacia						
1 per cent	1.0	0.005	2.147	2.142	0.057	2.37
3 per cent	3.0	0.013	2.186	2.173	0.088	3.61
5 per cent	5.0	0.025	2.221	2.196	0.111	4.50
7 per cent	7.0	0.034	2.254	2.220	0.135	5.42
10 per cent	10.0	0.048	2.294	2.246	0.161	6.39

This experiment may be performed by substituting a sol, containing a known quantity of powdered U. S. P. gum acacia (gum arabic) for the expressed plant sap. First determine the depression of the freezing point of a 5 per cent gum acacia sol, and then estimate it for a portion of the same solution, which contains 10.0 gm. of water, and to which 3.4224 gm. of sucrose have been added.

The percentage of bound water may be calculated by the following formula:

$$\frac{\Delta_a - (\Delta + K_m)}{\Delta_a - \Delta} \times 89.2,$$

where

Δ = the freezing point depression of the freshly expressed plant sap;

Δ_a = the freezing point depression after the addition of the sucrose;

$\Delta_a - \Delta$ = the actual additional depression due to the added sucrose;

K_m = the molecular constant for the depression of the freezing point of sucrose (2.085° C.);

$\Delta_a - (\Delta + K_m)$ = the amount by which the depression found on addition of the sucrose is in excess of that expected theoretically;

89.2 = the amount of actual free water when 100 gm. of water has dissolved 34.224 gm. of sucrose.

Results obtained from the use of the leaves of three varieties of winter wheat (*Triticum vulgare*), *Cereus*, a cactus-like plant, and artificial sols prepared from distilled water and U. S. P. gum acacia, are given in Table III.

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Expt. 71. Determination of Electrical Conductivity.—The measurement of the electrical conductivity of biological fluids is of importance since it serves as a measure of differentiation between the concentration

of ionized electrolytes and the total concentration of materials in solution. Whereas osmotic pressure measures the total concentration of both ions and molecules, electrical conductivity measurements serve to indicate the relative concentration of ionized electrolytes. The electrical current is carried through a solution only by ions. Water which is very poorly ionized $C_H = 10^{-7}$ or $C_{OH} = 10^{-7}$ (Expt. 72), is almost a non-conductor. When, however, a small amount of a strong acid or a strong base or a salt is added, it becomes a conductor of electricity and the solution is then known as an electrolyte.

The conductivity of a solution is measured indirectly rather than directly by determining the resistance of the solution to the passage of an electric current between two electrodes which are immersed in the solution. A diagram of the necessary apparatus is shown in Fig. 14.

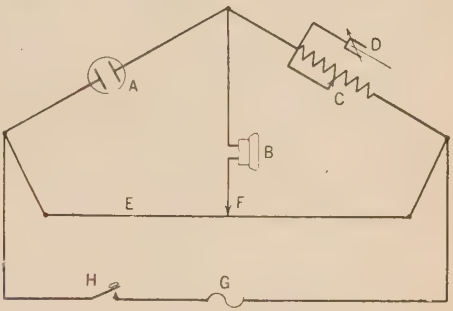

Fig. 14.

(*A*) is a conductivity cell containing the solution in which are immersed two electrodes, usually of platinum, coated with a coherent and uniform layer of platinum black.

(*B*) is a telephone which is used as a current detector. An alternating current galvanometer may be used in place of the telephone, providing that a slightly lower degree of accuracy is satisfactory.

(*C*) is a reistance box containing a series of resistance coils of known value. For plant sap work, a dial decade resistance box, having dials of $(10 \times 1) + (9 \times 10) + (9 \times 100)$ ohms is adequate.

(*D*) is an adjustable air condenser balanced across the terminals of the resistance box so as to annul the capacity of the cell (*A*). This may be omitted in approximate work.

(*E*) is an accurately calibrated slide wire and scale, the latter being graduated into 1000 or 10,000 divisions.

(*F*) is a sliding contact, connecting the telephone to the slide wire, and capable of being moved along it.

(*G*) is a source of high-frequency, alternating electric current. Many forms of apparatus have been devised for this purpose. The preferable form is a Vreeland Oscillator, if exact measurements are to be made. Satisfactory measurements may be made where a constant speed, high-frequency, 1000 cycle generator or a microphone hummer are employed. Even a small induction coil may be used for approximate work.

(*H*) is a telegraph key between the source of current and the bridge proper.

Electrical conductivity of a plant sap.—Place a portion of the plant sap (Expt. 68) in the conductivity cell. A Freas conductivity cell is suitable for this experiment. Make the proper connections, as shown in Fig. 14. Place the cell and contents in a thermostat and allow to come to 30° C. or to some predetermined temperature. Close the switch (*H*) and adjust the dials of the resistance box to a minimum tone in the telephone, keeping the sliding contact (*F*) exactly in the center of the slide wire (*E*). When this minimum tone is reached, move the sliding contact slowly along the slide wire until the tone disappears entirely, or until it reaches a minimum point beyond which it begins to increase. Having discovered an equal pitch of tones on either side of the minimum point, the true minimum is exactly halfway between these equal tone points. Record the reading of the resistance box and of the slide wire. Calculate the measured resistance of the electrolyte in the cell (*A*) in ohms according to the formula:

$$\text{Electrolyte Resistance} = R\left(\frac{a}{1000 - a}\right),$$

where R is the resistance in ohms, as recorded by the resistance box (*C*);
a is the reading of the slide wire scale;
$1000 - a$ is the portion of the slide wire nearest to the resistance box, assuming that the slide wire is graduated in 1000 divisions.

Conductivity (*K*) is the reciprocal of resistance

$$K = \frac{1}{R}.$$

Specific electrical conductivity (κ) is defined as the conductivity of a cube of an electrolyte, 1 cm. on an edge, placed between electrodes 1 cm. apart and of 1 sq. cm. area. Because the construction of such a cell, having electrodes of exactly this area and spaced at exactly this distance is extremely difficult, it is necessary to determine the *cell constant.* This is done by measuring the resistance of a solution of an electrolyte, the electrical conductivity of which is already known, and calculating from this a cell constant for each cell. Potassium chloride solutions are conveniently employed for this purpose. Table IV gives the constants for potassium chloride solutions of different concentrations and at different temperatures.

TABLE IV

SPECIFIC CONDUCTIVITY OF STANDARD KCl SOLUTIONS FOR DETERMINING THE RESISTANCE-CAPACITY OF CONDUCTIVITY VESSELS

t	KCl normal	KCl 1/10 normal	KCl 1/50 normal	KCl 1/100 normal
	κ	κ	κ	κ
15°	0.09252	0.01048	0.002243	0.001147
16	0.09441	0.01072	0.002294	0.001173
17	0.09631	0.01095	0.002345	0.001199
18	0.09822	0.01119	0.002397	0.001225
19	0.10014	0.01143	0.002449	0.001251
20	0.10207	0.01167	0.002501	0.001278
21	0.10400	0.01191	0.002553	0.001305
22	0.10594	0.01215	0.002606	0.001332
23	0.10789	0.01239	0.002659	0.001359
24	0.10984	0.01264	0.002712	0.001386
25	0.11180	0.01288	0.002765	0.001413
26	0.11377	0.01313	0.002819	0.001441
27	0.11574	0.01337	0.002873	0.001468
28	.....	0.01362	0.002927	0.001496
29	.....	0.01387	0.002981	0.001524
30	.....	0.01412	0.003036	0.001552

The cell constant is determined by the formula:

$$\text{Cell Constant} = R\left(\frac{a}{1000 - a}\right) \text{(Specific electrical conductivity of known KCl solution).}$$

Therefore, calculate the specific electrical conductivity of the plant sap (κ) by the formula:

$$\kappa = \left(\frac{1000 - a}{a \cdot R}\right) \text{(Cell Constant).}$$

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CHAPTER III

HYDROGEN-ION CONCENTRATION AND BUFFER ACTION

Expt. 72. Hydrogen-ion Concentration.—It is well known that pure water dissociates slightly into both hydrogen and hydroxyl ions, the former being characteristic of acids, and the latter, of bases. At equilibrium, the rate of formation of the ions from the water molecules is equal to the rate of recombination of the ions. The rate of formation of ions is proportional to the concentration of undissociated water in a given volume, while the rate of recombination is proportional to the product of the concentrations of the positive and negative ions present. The latter are equal in number in pure water. According to the Law of Mass Action at equilibrium, the equation may be written

$$\frac{[H^+] \times [OH^-]}{[HOH]} = k, \text{ a constant.}$$

Because of its very slight dissociation, the concentration of the undissociated water remains practically constant, so

$$[H^+] \times [OH^-] = K_w$$

in which K_w is known as the ionization constant of pure water. Several investigators have determined the value of this constant to be 10^{-14} at 22° C. If the temperature rises, more molecules dissociate into ions, but if it falls, more ions combine to form molecules. In a liter of pure water at 22° C. $[H^+]$ and $[OH^-]$ each have the value 10^{-7}, or expressed in terms of normality, the solution is either 0.0000001 N acid or alkaline. The value 10^{-7} represents the *true* or *absolute neutral point*. Therefore, solutions are *acid* when they contain hydrogen-ion concentrations greater, or hydroxyl-ion concentrations less than 10^{-7}; and solutions are *alkaline* when they contain hydrogen-ion concentrations less or hydroxyl-ion concentrations greater than 10^{-7}.

Thus a solution 0.0001 N or 10^{-4} with respect to hydrogen ions is 0.0000000001 N or 10^{-10} with respect to hydroxyl ions, and its reaction is acid; while a solution 0.00000001 N or 10^{-8} with respect to hydrogen ions is 0.000001 N or 10^{-6} with respect to hydroxyl ions, and the reaction is alkaline. So it is clear that, whatever the hydrogen-ion concentration when a substance is dissolved in water, the product of the concentration of the hydrogen and hydroxyl ions is always the sum of the exponents of 10 or 10^{-14}. It is worth noting that when the hydrogen-ion concentration is 10^{-14}, the hydroxyl-ion concentration is 10^{0} or normal. In consequence, to indicate acidity or alkalinity, it is necessary only to give the value of the hydrogen-ion concentration, for its corresponding hydroxyl-ion concentration follows directly as is shown above.

Actual and total acidity or alkalinity.—A 0.1 N solution of hydrochloric acid at 18° C. is ionized 92 per cent, while a 0.1 N solution of acetic acid has only 1.3 per cent of its available hydrogen ionized.[1] An acid, such as hydrochloric, which has a large proportion of its hydrogen atoms ionized, is regarded as a strong acid; while an acid, such as acetic, which has only a small proportion of its hydrogen atoms ionized, is considered a weak acid. That is, the *true* or *actual* acidity or the alkalinity of a solution depends on the hydrogen- or hydroxyl-ion concentration. A determination of the actual ionized hydrogen present gives a measurement of the hydrogen-ion concentration. It should be observed that there is a distinction between the *available* or *total* acidity or alkalinity, which is determined by titration with standard neutralizing solution, and the *actual* acidity or alkalinity.

Methods for determination.—There are two methods for the determination of hydrogen-ion concentration; they are the electrometric or potentiometer method, and the colorimetric or indicator method. The electrometric method depends upon measurement of the electromotive force of a system, in which are included (*a*) a saturated calomel electrode or a hydrogen electrode immersed in a solution of known hydrogen-ion concentration, (*b*) a hydrogen electrode surrounded by the solution whose hydrogen-ion concentration is to be determined, and (*c*) a potentiometer. A complete discussion and the directions for making the determination, are given in Clark's book. In 1909 Sörensen proposed that hydrogen-ion concentration be designated by the symbol pH.

[1] In this discussion the newer theories of complete ionization of electrolytes and of ion activity are not used. It is the feeling of the author that the purpose of this manual can be better served by using the older terminology, at least until the newer has been more widely taught.

The pH scale.—When determining the hydrogen-ion concentration by the electrometric method, the potentials of the hydrogen electrode are placed in an equation which reduces to the form:

$$\frac{\text{Potential}}{\text{Numerical factor}} = \log \frac{1}{[H^+]}.$$

According to Sörensen's suggestion, the expression, $\log \frac{1}{[H^+]}$, may be indicated by the symbol pH.

As an illustration of the method of converting $[H^+]$ into its corresponding pH value, the following example is given. Michaelis and Pechstein have shown that the iso-electric point of casein lies at the $[H^+]$ of 2.5×10^{-5} *N*. Since the logarithm of a fraction is equal to the logarithm of the numerator minus the logarithm of the denominator, the equation reads:

$$pH = \log \frac{1}{2.5 \times 10^{-5}} = \log 1 - \log (2.5 \times 10^{-5}).$$

Since $\log 1 = 0$, then $pH = -\log (2.5 \times 10^{-5})$,

or $-\log 2.5 - \log 10^{-5} = -0.40 + 5.00 = 4.6$ or $10^{-4.6}$.

That is

$$pH = 4.6.$$

To calculate the corresponding $[H^+]$ when the isoelectric point of tyrosine, pH = 5.41, is given, find the number corresponding to the negative logarithm of the pH value. That is

$$[H^+] = 10^{-5.41} = 10^{-6+0.59} = 10^{0.59} \times 10^{-6}$$

But $10^{0.59} = 3.89$ (antilog of 0.59).

Therefore pH 5.41 is equivalent to $[H^+] = 3.89 \times 10^{-6}$.

The investigations of Kendall show that distilled water, when in equilibrium with the carbon dioxide of the air in the laboratory, has a pH of about 5.7.

The relationship between hydrogen-ion concentration and pH is shown in Table V. Assuming that the hydrochloric acid and sodium hydroxide solutions are 100 per cent ionized, their normality may be considered identical with the hydrogen-ion concentration.

TABLE V

Normality	pH	$[H^+]$	$[OH^-]$
N HCl	0.0	1.0	10^{-14}
0.1 HCl	1.0	10^{-1}	10^{-13}
0.01 HCl	2.0	10^{-2}	10^{-12}
0.001 HCl	3.0	10^{-3}	10^{-11}
0.0001 HCl	4.0	10^{-4}	10^{-10}
0.00001 HCl	5.0	10^{-5}	10^{-9}
0.000001 HCl	6.0	10^{-6}	10^{-8}
Neutrality	7.0	10^{-7}	10^{-7}
0.000001 NaOH	8.0	10^{-8}	10^{-6}
0.00001 NaOH	9.0	10^{-9}	10^{-5}
0.0001 NaOH	10.0	10^{-10}	10^{-4}
0.001 NaOH	11.0	10^{-11}	10^{-3}
0.01 NaOH	12.0	10^{-12}	10^{-2}
0.1 NaOH	13.0	10^{-13}	10^{-1}
N NaOH	14.0	10^{-14}	1.0

The hydrogen-ion concentration and pH of some common solutions at 18° C., as recorded by Michaelis, are given in Table VI.

TABLE VI

	Normality	$[H^+]$	pH
HCl.....	1.0	8.0×10^{-1}	0.10
HCl.....	0.1	8.4×10^{-2}	1.071
HCl.....	0.01	9.5×10^{-3}	2.022
HCl.....	0.001	9.7×10^{-4}	3.013
HCl.....	0.0001	9.8×10^{-5}	4.009
CH_3COOH.....	1.0	4.3×10^{-3}	2.366
CH_3COOH.....	0.1	1.36×10^{-3}	2.866
CH_3COOH.....	0.01	4.3×10^{-4}	3.366
CH_3COOH.....	0.001	1.36×10^{-4}	3.866
NaOH.....	1.0	0.90×10^{-14}	14.05
NaOH.....	0.1	0.86×10^{-13}	13.07
NaOH.....	0.01	0.76×10^{-12}	12.12
NaOH.....	0.001	0.74×10^{-11}	11.13
NH_4OH.....	1.0	1.7×10^{-12}	11.77
NH_4OH.....	0.1	5.4×10^{-12}	11.27
NH_4OH.....	0.01	1.7×10^{-11}	10.77
NH_4OH.....	0.001	5.4×10^{-10}	10.27

Colorimetric method.—This method is not as accurate as the electrometric, but it is to be recommended for its convenience. Since all indicator ranges have been established by the electrometric method, it is a secondary rather than a primary method. It is based on the fact that each indicator has a characteristic zone of hydrogen-ion concentration within which its color changes occur. Table VII contains the list of sulphonphthalein series of indicators, which have been developed by Clark and Lubs. It includes the chemical and common names, the concentration of indicator convenient for use, a general indication of the color change, and the pH range.

TABLE VII

INDICATORS

Chemical Name	Common Name	Concentration	Color Change, Acid to Base	pH Range
		Per Cent		
Thymol sulfon phthalein..	Thymol blue	0.04	Red-yellow	1.2–2.8
Tetra bromo phenol sulfon phthalein.....	Brom phenol blue	0.04	Yellow-blue	3.0–4.6
Ortho carboxy benzene azo di methyl aniline.....	Methyl red	0.02	Red-yellow	4.4–6.0
Di bromo ortho cresol sulfon phthalein.....	Brom cresol purple	0.04	Yellow-purple	5.2–6.8
Di bromo thymol sulfon phthalein.....	Brom thymol blue	0.04	Yellow-blue	6.0–7.6
Phenol sulfon phthalein...	Phenol red	0.02	Yellow-red	6.8–8.4
Ortho cresol sulfon phthalein	Cresol red	0.02	Yellow-red	7.2–8.8
Thymol sulfon phthalein..	Thymol blue	0.04	Yellow-blue	8.0–9.6
Ortho cresol phthalein....	Cresol phthalein	0.02	Colorless-red	8.2–9.8

From W. M. Clark, "The Determination of Hydrogen Ions," Williams & Wilkins Company, Baltimore. 1920. By Permission.

Figure 15 shows the per cent dissociation of the indicators and their significant pH values.

Alcohol solutions of the indicators may be used when the alcohol is not objectionable and when the acidity of the free acid dye in the indicator solution does not interfere with accuracy; however, Clark and Lubs prefer aqueous solutions of the alkali salts, prepared as concentrated stock solutions to be diluted as needed.

Prepare *stock* solutions of indicators as follows: Grind 0.1 gm. of the

dry powder in an agate mortar with the corresponding quantity of 0.05 *N* sodium hydroxide solution as given in Table VIII.

TABLE VIII

Molecular Weight	Indicator	0.05 *N* NaOH per Decigram
466.30	Thymol blue	4.3 cc.
669.82	Brom phenol blue	3.0 cc.
269.12	Methyl red	7.4 cc.
540.01	Brom cresol purple	3.7 cc.
624.12	Brom thymol blue	3.2 cc.
354.17	Phenol red	5.7 cc.
382.17	Cresol red	5.3 cc.

When solution is complete, dilute to 25 cc. with distilled water. This results in a 0.4 per cent solution of the original indicator. To prepare the concentrations indicated in Table VII, dilute 5 cc. each of thymol blue, brom phenol blue, brom cresol purple, and brom thymol blue, and 2.5 cc. each of methyl red, phenol red, and cresol red, to 50 cc. Prepare ortho cresol phthalein by dissolving 0.04 gm. in 100 cc. of 95 per cent ethyl alcohol.

When using an indicator, add 5 drops to 10 cc. of the solution to be tested, and compare the color with that developed by the addition of a similar amount of indicator to 10 cc. portions of standard buffer solutions.

Buffer action and buffer solutions.— Van Slyke defines buffers as "substances which by their presence in solution increase the amount of acid or alkali that must be added to cause unit change in pH." A buffer solution generally consists of a mixture of an acid with its alkali salt. He has adopted a unit for the measurement of buffer values. "A solution has a buffer value of 1 when a liter will take up 1 gram

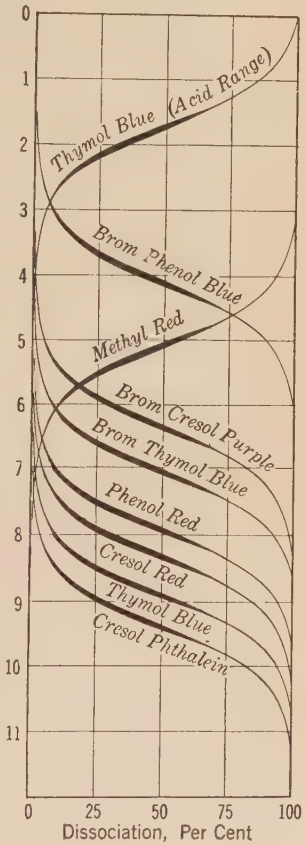

FIG. 15.

equivalent of strong acid or alkali per unit change in pH." Expressed specifically, each cubic centimeter of normal alkali or acid that must be added to a liter of solution to raise or lower its pH by 1, increases the buffer value by 0.001. If one solution has twice the buffer value of another solution, twice as much base or acid is required to change the pH by a given amount.

Buffer action may be illustrated as follows: In a solution of acetic acid there is, according to the Law of Mass Action, an equilibrium between the ions and the undissociated molecules, which may be represented by the equation

$$\frac{[H^+] \times [C_2H_3O_2^-]}{[HC_2H_3O_2]} = K.$$

The value of K is constant whatever the molar concentration of the solution. If more acetic acid is added to the solution, the concentration of acetic acid molecules is increased, and part of the acetic acid dissociates and produces greater concentrations of both hydrogen and acetate ions, in order that equilibrium may be maintained. The value of K also remains the same even when another substance with a common ion, such as sodium acetate, is present. For example, a molar solution of acetic acid is ionized 0.4 per cent, while a molar solution of sodium acetate is ionized 53 per cent. If to this molar solution of acetic acid there is added sufficient sodium acetate to make the solution molar in respect to the latter substance, also, the concentration of the acetate ions from both sources will be $0.53 + 0.004 = 0.534$ or nearly 134 times that in the acid alone. Consequently, to maintain equilibrium the hydrogen ions are diminished $\frac{1}{134}$ of their former concentration. That is, the addition of sodium acetate causes a reduction of the concentration of hydrogen ions and it therefore serves as a buffer. Because of the presence of the buffer in the solution, a quantity of alkali quite out of proportion to the amount of acetic acid must be added to cause unit change in pH. What will be the effect of dilution upon the pH value of such an acetic acid-sodium acetate mixture?

Clark and Lubs recommend the preparation of buffer solutions having intervals of 0.2 pH; these are given in Table IX.

Prepare the various mixtures from the following stock solutions: 0.2 M potassium chloride, 0.2 M acid potassium phosphate, 0.2 M acid potassium phthalate, 0.2 M boric acid with 0.2 M potassium chloride, 0.2 M sodium hydroxide, and 0.2 M hydrochloric acid. For the method of their preparation, see Clark, pp. 99–107.

The instructor should prepare several of these standard buffer solutions, and should check them by the electrometric method. The

following are suggested as desirable pH ranges: 1.4, 2.8, 4.4, 4.6, 5.4, 7.0, 8.0, 8.6, and 9.6.

Determination of pH by the colorimetric method.—Give each student a series of the standard buffer solutions as unknowns. Let him determine the approximate pH of each solution by finding the limits characteristic of the indicator. Using uniform test tubes, test 10 cc. portions of the solution to be examined, by adding 5 drops of each of the

TABLE IX

Composition of Mixtures Giving pH Values at 20° C. at Intervals of 0.2

pH			
KCl–HCl Mixtures			
1.2	50 cc. 0.2 *M* KCl	64.5 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.4	50 cc. 0.2 *M* KCl	41.5 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.6	50 cc. 0.2 *M* KCl	26.3 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.8	50 cc. 0.2 *M* KCl	16.6 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.0	50 cc. 0.2 *M* KCl	10.6 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.2	50 cc. 0.2 *M* KCl	6.7 cc. 0.2 *M* HCl	Dilute to 200 cc.
Phthalate–HCl Mixtures			
2.2	50 cc. 0.2 *M* KHPhthalate	46.70 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.4	50 cc. 0.2 *M* KHPhthalate	39.60 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.6	50 cc. 0.2 *M* KHPhthalate	32.95 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.8	50 cc. 0.2 *M* KHPhthalate	26.42 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.0	50 cc. 0.2 *M* KHPhthalate	20.32 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.2	50 cc. 0.2 *M* KHPhthalate	14.70 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.4	50 cc. 0.2 *M* KHPhthalate	9.90 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.6	50 cc. 0.2 *M* KHPhthalate	5.97 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.8	50 cc. 0.2 *M* KHPhthalate	2.63 cc. 0.2 *M* HCl	Dilute to 200 cc.
Phthalate–NaOH Mixtures			
4.0	50 cc. 0.2 *M* KHPhthalate	0.40 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.2	50 cc. 0.2 *M* KHPhthalate	3.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.4	50 cc. 0.2 *M* KHPhthalate	7.50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.6	50 cc. 0.2 *M* KHPhthalate	12.15 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.8	50 cc. 0.2 *M* KHPhthalate	17.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.0	50 cc. 0.2 *M* KHPhthalate	23.85 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.2	50 cc. 0.2 *M* KHPhthalate	29.95 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.4	50 cc. 0.2 *M* KHPhthalate	35.45 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.6	50 cc. 0.2 *M* KHPhthalate	39.85 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.8	50 cc. 0.2 *M* KHPhthalate	43.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.0	50 cc. 0.2 *M* KHPhthalate	45.45 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.2	50 cc. 0.2 *M* KHPhthalate	47.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.

TABLE IX—*Continued*

KH_2PO_4–NaOH Mixtures

pH			
5.8	50 cc. 0.2 *M* KH_2PO_4	3.72 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.0	50 cc. 0.2 *M* KH_2PO_4	5.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.2	50 cc. 0.2 *M* KH_2PO_4	8.60 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.4	50 cc. 0.2 *M* KH_2PO_4	12.60 cc. 0.2 *M* NaOH	Dilute to 200 c.c
6.6	50 cc. 0.2 *M* KH_2PO_4	17.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.8	50 cc. 0.2 *M* KH_2PO_4	23.65 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.0	50 cc. 0.2 *M* KH_2PO_4	29.63 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.2	50 cc. 0.2 *M* KH_2PO_4	35.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.4	50 cc. 0.2 *M* KH_2PO_4	39.50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.6	50 cc. 0.2 *M* KH_2PO_4	42.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.8	50 cc. 0.2 *M* KH_2PO_4	45.20 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.0	50 cc. 0.2 *M* KH_2PO_4	46.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.

Boric Acid, KCl–NaOH Mixtures

7.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	2.61 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	3.97 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.2	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	5.90 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.4	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	8.50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.6	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	12.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	16.30 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	21.30 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.2	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	26.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.4	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	32.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.6	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	36.85 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	40.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
10.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	43.90 cc. 0.2 *M* NaOH	Dilute to 200 cc.

From W. M. Clark, "The Determination of Hydrogen Ions." Williams & Wilkins Company, Baltimore, 1920. By Permission.

indicators in the series, beginning with thymol blue. If, after thorough mixing, this indicator is transformed completely to its acid color by the solution, try the indicator next in the series. Continue this process until the indicator, which shows that the solution has a pH range within its characteristic limits, is found. From the range of color transformation produced in the indicator, estimate the approximate pH of the unknown. This value may be determined by a comparison of the colors produced, with those in the color chart,[2] Fig. 8, in Clark.

[2] Separates of the color chart may be obtained from Williams & Wilkins Company Baltimore, Md.

To determine more accurately the hydrogen-ion concentration, compare the color of the unknown solution with standard buffer solutions to which the same indicator has been added. For example, if in the preliminary test of a given solution, phenol red is not changed either to a bright yellow or to a full red, but its proportion of red is low, a pH of about 7.0 is indicated. Therefore, those buffer solutions having pH values near 7.0 should be used for comparison. Place in uniform test tubes 10 cc. of several of the KH_2PO_4–NaOH buffer solutions, Table IX, and to each tube add 5 drops of phenol red. Mix thoroughly, and note the graded series of colors. Observe where the color of the unknown solution and the standard match, and record the results.

Sources of error.—There are two chief sources of error in the colorimetric determination of hydrogen ions, the *protein* and the *salt*. If there are present in the solution being tested, substances which absorb the indicator or otherwise remove it from the field of action, the equilibria upon which the color depends may be altered. An indicator in such a solution may show a color intensity or even a color quality, but these will differ greatly from the color intensity of a solution having the same pH and the same concentration of indicator but no disturbing factors. Protein solutions frequently produce such errors; they are generally greater the more complex and concentrated the proteins, and they decrease as the degree of hydrolysis increases. The presence of inorganic salts may also affect the equilibria so that different intensities and shades of color are produced. A neutral salt, such as sodium chloride, may cause these errors.

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Expt. 73. Buffer Action of Wheat Flour Extracts.—Suspend 20 gm. each of a patent and of a clear wheat (*Triticum vulgare*) flour (Expt. 32, footnote) in 100 cc. of redistilled water at 25° C. Extract for one hour in a water thermostat at this temperature, shaking every 10 minutes to keep the flour particles in suspension. At the end of the hour, centrifuge until all of the suspended matter has been thrown down, and decant the clear supernatant liquid. The water extract of a wheat flour contains a variety of soluble organic and inorganic substances (Expt. 32). Using two Erlenmeyer flasks, pipet into one 50 cc. of the patent flour extract, and into the other a similar quantity of the extract of the clear flour. Add to each 5 cc. of a 0.02 *N* sodium hydroxide solution, which has been freshly prepared from a 0.2 *N* solution, and mix thoroughly. Determine the hydrogen-ion concentration of each mixture by the

colorimetric method (Expt. 72). For this purpose use a comparator, such as that described by Cooledge for comparing standard and unknown solutions. A diagram of this device is illustrated in Fig. 16. The standard buffer solutions used have a pH range from 6.8 to 8.4 with an interval of 0.1 pH for each color shade in the series. The same indicator must be used for both standard and unknown solutions. To prepare the *unknown-with-indicator* solution, pipet into uniform test tubes 25 cc. of the alkali mixture of each flour extract and add to each 0.4 cc. of a 0.05 per cent solution of phenol red. The remaining portion of the alkali mixture of each extract should be used as the *unknown-without-indicator*. In the case of wheat flour extracts, it is possible to use either the original water extract or the extract to which alkali has been added, as the unknown-without-indicator; but if a plant sap containing substances affected by alkali is used, alkali must always be added before making color comparisons. The original water extract of patent flour has a pH range of 5.9 to 6.1, while that of a clear flour is from 6.2 to 6.4. Compare the hydrogen-ion concentrations of the original water extracts with those of the extracts treated with dilute alkali, and record results. To what substances in each extract is the difference in buffer action due? This method of determining buffer action may be used as a basis for establishing the grade of wheat flour.

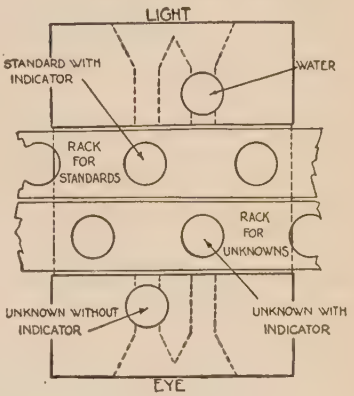

FIG. 16.

0.2 *N sodium hydroxide solution.*—This solution should be as free as possible from carbonate. Place 100 gm. of high grade sodium hydroxide in a Pyrex Erlenmeyer flask and dissolve it in 100 cc. of distilled water. Cover the mouth of the flask with tin foil and allow the solution to stand over night until most of the carbonate has settled. Siphon or pipet off the clear supernatant liquid and dilute quickly with carbon dioxide free distilled water to a concentration of slightly more than 0.1 *N*. Withdraw 10 cc. of this solution and standardize it roughly against an acid solution of known normality. From this approximate standardization calculate the dilution required to make a 0.2 *N* solution. Make the required dilution with the least possible exposure to the air and preserve in a *paraffined* bottle. The solution should now be carefully standardardized.

Phenol red (phenol sulfon phthalein) *solution.*—This may be obtained

in solution in physician's ampules and should be diluted to a concentration of 0.05 per cent.

Preparation of colorimetric standards.—Prepare standard buffer solutions of KH_2PO_4–NaOH mixtures, and of boric acid, KCl–NaOH mixtures (Table IX, p. 100), which have a pH range from 6.8 to 8.4 with an interval of 0.1 pH. Check these by means of the electrometric method, described by Clark. Place the standardized buffer solutions in a series of uniform hard glass test tubes, or other suitable containers; add to each solution 5 drops of phenol red, and a drop of toluene as a protection against the growth of mold. Seal by drawing off the tubes in a flame, and preserve in the dark when not in use. Though reasonably permanent, these color standards may change, so they should be used with caution.

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CHAPTER IV

PROTEINS

I. Nitrogen in Organic Compounds

Expt. 74. Test for Nitrogen.—When an organic nitrogenous substance is heated with metallic sodium or potassium, the nitrogen is converted into the corresponding sodium or potassium cyanide by the action of the alkali metal and carbon. Support a clean, dry Pyrex test tube, 100 × 14 mm., in a vertical position by passing it through a hole in an asbestos board in such a way as to hold it by the flare of the top. Place in the tube a small piece of bright metallic sodium, about the size of a pea and free from adhering oil, and heat with a very low flame until the sodium melts and a layer of sodium vapor about 1 cm. deep is formed. Drop a small portion of a protein, such as casein, into the tube without touching the walls. Continue heating while decomposition is taking place, but do not drive the vapors outside the tube by heating too much. After the action has ceased, heat until the contents become dull red and then cool to room temperature. Add two or three drops of ethyl alcohol to destroy any unused sodium and stir with a glass rod to break up the charred mass. As soon as the reaction between the sodium and ethyl alcohol has ceased, *cautiously* add a drop or two of distilled water. When it is certain that the sodium has been destroyed, again add a few drops of distilled water and stir. Heat the mixture to boiling, filter through a *small* filter paper, and rinse the tube with three or four portions of distilled water so that the total volume of the filtrate will not be more than 3 cc. The filtrate should be practically colorless if the fusion has been satisfactory. If the filtrate is not already alkaline, make it so by the addition of dilute sodium hydroxide solution.

The method of testing for the presence of the cyanide is that developed by Vichoever and Johns. To the filtrate contained in a test tube, add 2 or 3 drops of a freshly prepared 3 per cent ferrous sulfate solution and about 50 mg. of potassium fluoride. The latter is more efficient than any other salt in hastening the formation of Prussian blue, but its action is not definitely known. Close the tube with a stopper to keep

out the air and rotate the contents just enough to mix the reagents, but avoid excessive shaking so that the ferrous hydroxide will not be oxidized. Then allow the mixture to stand 5 to 10 minutes. Acidify the solution with 30 per cent nitric acid or with approximately *N* sulfuric acid. Test with litmus paper to be sure that it is acid. The formation of Prussian blue indicates the presence of nitrogen. The mixture may be distinctly blue without the separation of a precipitate; but the latter may separate, if allowed to stand for some time. Under what conditions is Prussian blue obtained in the colloidal state? The solution is acidified with nitric or sulfuric acid instead of hydrochloric because an excess of this acid results in the formation of ferric chloride, which tends to make the color of the suspension green; that is, the mixture of the yellow ferric chloride solution with the Prussian blue produces the green shade. Write equations for the reactions that take place in this test. The test is applicable to all naturally occurring organic nitrogenous compounds.

*Mulliken, S. P. A method for the indentification of pure organic compounds. Vol. 1, pp. 10–12. John Wiley & Sons, New York, 1905.

*Viehoever, A., and Johns, C. O. On the determination of small quantities of hydrogen cyanide. *J. Am. Chem. Soc.*, 37, 601–607 (1915).

*Hostetter, J. C., and Roberts, H. S. Electrometric titrations, with special reference to the determinations of ferrous and ferric iron. *J. Am. Chem. Soc.*, 41, 1337–1357. Cf. 1353 (1919).

II. Precipitation and Coagulation of Proteins

The terms precipitation and coagulation are used indiscriminately by many investigators to describe the methods of bringing both colloids and proteins out of solution. However, Hardy has demonstrated that there is a distinct difference between them, and in the study of proteins the following differentiation is made. Precipitation is considered to be dependent on the ionization of the protein and is brought about by a very low concentration of the added electrolyte. Coagulation is dependent on the dehydration of the protein; for this purpose its ionization is unnecessary, but high concentrations of salts are required. They render the proteins insoluble, because the salt has greater affinity for water than that possessed by the protein. The same results are brought about also by heat and non-electrolytes, such as alcohol. Posternak has shown that the order of effectiveness of different inorganic salts in bringing about coagulation is the reverse in the case of *electronegative* and *electropositive* proteins.

Posternak, S. Contribution a l'étude causale des modifications d'état des colloids. Sur les propriétés physiques de la micelle albuminoide. *Ann. l'inst. Pasteur*, 15, 85–120, 169–208, 451–496, 570–592 (1901).

Hardy, W. B. Colloidal solution. The globulins. *J. Physiol.*, 33, 251–337 (1906–07).

Pauli, W. Untersuchungen über physikalische Zustandsänderungen der Kolloide. Fünfte Mitteilung. Die elektrische Ladung von Eiweiss. *Beitr. Chem. Physiol. Path.*, 7, 531–547 (1906).

Robertson, T. B. The physical chemistry of the proteins. pp. 107–136. Longmans, Green and Company, New York, 1918.

Expt. 75. Precipitation and Coagulation of Egg Albumin.—The coagulation of proteins includes two distinct processes: denaturation or dehydration and subsequent precipitation or flocculation. In the former, the character of the protein is greatly altered; that is, it is so changed from its hydrophilic colloid form that it acquires many of the properties of a hydrophobic colloid, and may therefore be precipitated. Sörensen shows that in the denaturation of egg albumin either by heat or by alcohol, water is removed but its removal is not complete, for denatured egg albumin always contains some water. The denaturation of a protein may be accomplished either by raising the temperature, or by increasing the hydrogen- or hydroxyl-ion concentration or by both; or it may be done by adding alcohol. In general, acids are more effective than alkalis. The precipitation of the denatured protein is more rapid and complete, the more nearly the concentration of hydrogen ions approaches the isoelectric point.

Place in each of a series of 12 numbered test tubes 5 cc. of egg albumin solution; to tube 1 add 15 cc. of distilled water; to the other tubes add 15 cc. of a mixture of distilled water and 0.01 *N* hydrochloric acid, the amount of the former being decreased as the latter is increased until tube 12 receives 15 cc. of 0.01 *N* hydrochloric acid. The amounts of acid which should be added, beginning with tube two, are as follows: 0.75 cc., 1.50 cc., 2.25 cc., 3.00 cc., 3.75 cc., 5.62 cc., 7.50 cc., 9.37 cc., 11.25 cc., 13.13 cc., and 15.00 cc. Shake each test tube thoroughly and then immerse in a water bath at 64–65° C., using as a support a copper plate perforated for test tubes. Heat for 15 minutes. At the end of this time the contents of tubes 1–3 will vary from clear to opalescent; tubes 6–9 should contain precipitate, the largest amount being in tube 8; in the remaining tubes, there should be no precipitate, only an opalescence which should decrease to tube 12.

Filter the contents of each tube through dry filter paper. To produce flocculation, bring all the filtrates to the optimum hydrogen-ion concentration, pH 4.7–4.8, by the addition of 7.5 cc. of the buffer mixture for each 15 cc. of the filtrate. A precipitate appears in all the tubes except 6–9; this increases in quantity with the increasing acidity of the original solution. Only the egg albumin which has been completely denatured is precipitated. To precipitate any still undena-

tured, again filter the contents of each tube and heat the filtrates for 15 minutes in the water bath at 64–65° C. It will then be observed that all the tubes contain precipitates; that in the group 1–6, 1 contains most and 6 least; and that, in the other group, the amount decreases from 7–12.

For this experiment, Sörensen recommends the use of 5 cc. of a pure egg albumin solution, prepared from recrystallized egg albumin containing 40–50 mg. of nitrogen; but the fundamental principles may be shown by using unpurified egg albumin. Since the hydrogen-ion concentration of unpurified egg albumin varies with the freshness of the eggs, the amounts of precipitate obtained in the different tubes by the denaturation process may vary from those suggested here.

Egg albumin solution.—Dilute one volume of fresh egg-white with three volumes of distilled water, mix thoroughly, and filter to remove ovoglobulin.

Buffer solution.—Mix equal volumes of N sodium acetate and N acetic acid. This solution has a pH 4.7–4.8.

Sörensen, S. P. L. Studies on proteins. *Compt. rend. trav. lab. Carlsberg*, 12, 1–11 (1915).

Sörensen, S. P. L., and Höyrup, Margrethe. Studies on proteins. I. On the preparation of egg-albumin solutions of well-defined composition, and on the analytical methods used. *Compt. rend. trav. lab. Carlsberg*, 12, 12–67 (1916).

Sörensen, S. P. L., with Höyrup, Margrethe, Hempel, Jenny, and Palitzsch, S. Studies on proteins. II. On the capacity of egg-albumin to combine with acids or bases. *Compt. rend. trav. lab. Carlsberg*, 12, 68–163 (1917).

Sörensen, S. P. L., and Höyrup, Margrethe. Studies on proteins. III. On the composition and properties of egg-albumin separated in crystalline form by means of ammonium sulphate. *Compt. rend. trav. lab. Carlsberg*, 12, 164–212 (1917).

Sörensen, S. P. L., and Höyrup, Margrethe. Studies on proteins. IV. On the state of equilibrium between crystallized egg-albumin and surrounding mother liquor, and on the applicability of Gibbs' phase rule to such systems. *Compt. rend. trav. lab. Carlsberg*, 12, 213–261 (1917).

Sörensen, S. P. L., with Christiansen, J. A., Höyrup, Margrethe, Goldschmidt, S., and Palitzsch, S. Studies on proteins. V. On the osmotic pressure of egg-albumin solutions. *Compt. rend. trav. lab. Carlsberg*, 12, 262–372 (1917).

Sörensen, S. P. L., and Palitzsch, S. Studies on proteins. VI. On crystalline egg-albumin salts, precipitated by salts other than sulphate of ammonia. *Compt. rend. trav. lab. Carlsberg*, 15, No. 2, 1–10 (1923).

Sörensen, Margrethe and S. P. L. Studies on proteins. VII. On the coagulation of proteins by heating. *Compt. rend. trav. lab. Carlsberg*, 15, No. 9, 1–26 (1924).

Healy, D. J., and Peter, A. M. The hydrogen ion concentrations and basicity of egg yolk and egg white. *Am. J. Physiol.*, 74, 363–368 (1925),

III. Color Reactions of the Proteins

Not all proteins respond to all of the color tests, for the colors produced are due to the presence of certain specific chemical groups, which may or may not be present in a particular protein. Every protein will give, however, a positive reaction in a large number of the tests. Various non-protein substances also react to certain of these tests.

A plant protein that responds to all of the tests is obtained from the soy bean (*Glycine hispida*). The water extract of soy bean meal contains about 16 per cent of the globulin, glycinin, besides a small amount of other proteins. A mixture of 10 gm. of soy bean meal and 100 cc. of distilled water, allowed to stand one hour with occasional stirring and then filtered, gives a good extract for use in the tests. The extraction of glycinin by water is doubtless due to the presence of acid potassium salts of phosphorus and the organic acids contained in the seeds. This has been pointed out by Osborne and Campbell in the case of the globulin, legumin, in the seeds of the pea, lentil, horse bean, and vetch. The water extract gives the Molisch test, but the carbohydrate group is not necessarily present in the protein molecule. A protein that responds to all of the tests from the animal standpoint is egg albumin. To prepare a solution for this purpose, dilute one volume of fresh egg white with four volumes of distilled water; mix thoroughly, and filter the solution to remove the ovoglobulin.

Osborne, T. B., and Campbell, G. F. The proteids of the pea, lentil, horse bean, and vetch. *J. Am. Chem. Soc.*, 20, 410–419 (1898).

Osborne, T. B., and Campbell, G. F. Proteids of the soy bean. *J. Am. Chem. Soc.*, 20, 419–428 (1898).

Expt. 76. Biuret Test.—To 2–3 cc. of the protein solution add, drop by drop with constant shaking, the biuret reagent until a pink or violet color develops. If the color does not appear quickly, allow the solution to stand 15–20 minutes. A protein solution results in a reddish- or bluish-violet except in the case of protamines, which yield a pink color; while the derived proteins such as proteoses and peptones develop pink. Again perform the test, using 3 cc. of Witte's peptone solution and adding 2 cc. of the biuret reagent. If considerable ammonium salt should be present, it will interfere with the test, so that it is necessary to add strong sodium hydroxide solution until the salt has been decomposed. To illustrate this, add to the peptone test 2 cc. of a saturated ammonium sulfate solution, and observe that the pink color disappears; then add a 30 per cent sodium hydroixde solution until the pink color returns. To what chemical grouping in the protein molecule is the

biuret test due? Is it a general or a specific protein test? What is the source of the term biuret? This test may be used to determine the completion of a protein hydrolysis; the reaction will then be entirely negative.

Biuret reagent.—To 1000 cc. of 10 per cent sodium hydroxide solution, add 25 cc. of a 3 per cent copper sulfate solution, a few cubic centimeters at a time, stirring constantly. A blue solution results. Glass wool may be used to filter the reagent if filtration is necessary.

Witte's peptone solution.—Add 2 gm. of Witte's peptone to 100 cc. of distilled water, and warm gently to dissolve it; just acidify with acetic acid and filter.

Schiff, H. Biuretreactionen. *Ber.*, 29, 298–303 (1896).
Schiff, H. Ueber Biuret und Biuretreaction. *Ann.*, 299, 236–266 (1897).
Schiff, H. Trennung von Amin- und Säurefunction in Lösungen von Eiweisskörpern. *Ann.*, 319, 287–303. Cf. 300–303 (1901).
*Kantor, J. L., and Gies, W. J. Methods of applying the biuret test. *Biochem. Bull.*, 1, 264–269 (1911).

Expt. 77. Ninhydrin Test.—Place in a test tube 1 cc. of the protein solution, 1 cc. of a 10 per cent pyridine solution, and 1 cc. of freshly prepared ninhydrin solution; heat the mixture in a rapidly boiling water bath for 20 minutes, and then cool. If the test is positive, an intense blue or bluish-violet color develops. The test gives positive results with proteins, peptones, peptides, and amino acids which contain a free carboxyl and α-amino group. Since this is also a general reaction for ammonium salts, as well as for certain amines and amides, care must be taken to avoid their presence. Absolute cleanliness is essential to the success of the test. The original protein solution should be neutral to phenolphthalein. It seems probable that, because of its buffer value, the base, pyridine, maintains a constant hydrogen-ion concentration.

Ninhydrin reagent (triketohydrindene hydrate).—Dissolve 0.1 gm. of triketohydrindene hydrate in 50 cc. of distilled water.

Ruhemann, S. Triketohydrindene hydrate. *J. Chem. Soc.*, 97, 2025–2031 (1910). The author discovered this reaction.
Abderhalden, E., und Kiutsi, M. Biologische Untersuchungen über Schwangerschaft. Die Diagnose der Schwangerschaft mittels der optischen Methode und dem Dialysierverfahren. *Z. physiol. Chem.*, 77, 249–258 (1912).
Abderhalden, E. Weiterer Beitrag zur biologischen Festellung der Schwangerschaft. *Z. physiol. Chem.*, 81, 90–98 (1912).
Abderhalden, E., und Schmidt, H. Einige Beobachtungen und Versuche mit Triketohydrindenhydrat. (Ruhemann.) *Z. physiol. Chem.*, 85, 143–147. Cf. 146 (1913). The authors demonstrate that the various amino acids do not show the same sensitivity to ninhydrin.

*Harding, V. J., and MacLean, R. M. A colorimetric method for the estimation of amino-acid α-nitrogen. *J. Biol. Chem.*, 20, 217–230. Cf. 226 (1915).
Harding, V. J., and Warneford, F. H. S. The ninhydrin reaction with amino-acids and ammonium salts. *J. Biol. Chem.*, 25, 319–335 (1916).
Harding, V. J., and MacLean, R. M. The ninhydrin reaction with amines and amides. *J. Biol. Chem.*, 25, 337–350 (1916).

Expt. 78. Millon's Test.—To 4–5 cc. of the protein solution, add a few drops of Millon's reagent. A white precipitate usually forms. Heat in a boiling water bath, and observe that the precipitate slowly turns pink or red or disappears, leaving a red solution. This test is particularly satisfactory for use with solid proteins. If the original protein solution is strongly alkaline, it should be neutralized because the alkali will precipitate the yellow or black oxides of mercury. The test is not satisfactory for use in solutions containing inorganic salts, which would precipitate the mercury present in the reagent. It is characteristic of all aromatic substances which contain a monohydroxybenzene nucleus. Hence it is given by phenol, salicylic acid, and many other substances. To what amino acid in the protein molecule is the reaction due? Again perform the test, using a 2 per cent gelatin sol and adding a mere trace of Millon's reagent from the end of a stirring rod. A pink solution results.

Millon's reagent.—Treat one part by weight of mercury with two parts by weight of nitric acid of sp. gr. 1.42, and warm gently until solution is complete. Dilute the resulting solution with two volumes of distilled water and allow to stand for several hours. Decant the supernatant liquid from the crystalline precipitate. The reagent contains mercurous and mercuric nitrates, together with an excess of nitric acid and a little nitrous acid.

*Millon, M. E. Sur un réactif propre aux composés protéiques. *Compt. rend.*, 28, 40–42 (1849).
Salkowski, E. Kleinere Mittheilungen. *Z. physiol. Chem.*, 12, 211–228 (1888).
Vaubel, W. Zur Kenntniss der Millon'schen Reaction. *Z. angew. Chem.*, 1125–1130 (1900).
Nasse, O. Über die Verwendbarkeit des Millon'schen Reagens. *Arch. ges. Physiol. (Pflüger's)*, 83, 361–368 (1901).

Expt. 79. Xanthoproteic Test.—To 3 cc. of the protein solution add about 1 cc. of concentrated nitric acid. A white precipitate forms, which upon boiling, turns yellow and may partly dissolve to give a yellow solution. Cool, and then carefully add 30 per cent sodium hydroxide until the solution is slightly alkaline. The color should change to deep orange. Again perform the test, using powdered gelatin. The reaction is due to the presence of a benzene nucleus in the

protein molecule, and the yellow color is the result of the formation of a nitro-benzene derivative. To what amino acids in the protein molecule is this test due?

*Salkowski, E. Kleinere Mittheilungen. *Z. physiol. Chem.*, 12, 211–228. Cf. 218–220 (1888).

Expt. 80. Loosely Bound Sulfur Test.—To 3 cc. of the protein solution, add an equal volume of 30 per cent sodium hydroxide solution and a few drops of saturated lead acetate solution. Boil the mixture for two or three minutes. A brown or black color develops and ultimately a black precipitate of lead sulfide separates. The reaction is due to the formation of sodium sulfide by the action of the strong alkali on the sulfur of the protein. What amino acids contain sulfur?

*Fleitmann, T. Ueber die Existenz eines schwefelfreien Proteins. *Ann. Chem. Pharm.*, 61, 121–126 (1847).

Fleitmann, T. Bestimmungen des Verhaltnisses, in welchem der Schwefel in seinen zwei verscheidenen Formen in den schwefel- und stickstoffhaltigen organischen Verbindungen erhalten ist. *Ann. Chem. Pharm.*, 66, 380–381 (1848).

*Osborne, T. B. Sulfur in protein bodies. *Conn. Agr. Expt. Sta., Report.* pp. 443–471, 1900; reprinted in *J. Am. Chem. Soc.*, 24, 140–167 (1902).

Johnson, T. B. Sulfur linkages in proteins. *J. Biol. Chem.*, 9, 439–448 (1911).

Expt. 81. Rosenheim's Formaldehyde Test.—To 2 or 3 cc. of the protein solution add 3 or 4 drops of formaldehyde solution (1 : 2500) and mix thoroughly. After two or three minutes carefully pour a little concentrated sulfuric acid down the side of the test tube so that the two liquids do not mix. If the test is positive, a violet or purple ring will form in about 5 minutes. Perform this test also with a 2 per cent gelatin sol. Rosenheim found that an oxidizing agent is necessary for the reaction; this is supplied by the sulfuric acid. Ordinarily the acid contains sufficient oxidizing agent, but if trouble is experienced, a small quantity of ferric chloride (5 mg. to 100 cc. of acid) should be added. The color developed is due to the condensation of the aldehyde with the α-position of the indole nucleus present in the protein molecule. What amino acid contains the indole nucleus? What is the principle of the Adamkiewicz reaction? Explain the detection of formaldehyde in milk. What is the Hopkins-Cole reaction?

*Adamkiewicz, A. Farbenreactionen des Albumin. *Arch. ges. Physiol.* (*Pflüger's*), 9, 156–162 (1874).

Adamkiewicz, A. Farbenreactionen des Albumin. *Arch. expt. Path.*, 3, 412–426 (1875).

Hopkins, F. G., and Cole, S. W. On the proteid reaction of Adamkiewicz, with contributions to the chemistry of glyoxylic acid. *Proc. Roy. Soc.* (*London*), 68, 21–23. Cf. 32 (1901).

*Rosenheim, O. A color reaction of formaldehyde with proteids and its relation to the Adamkiewicz reaction. *Biochem. J.*, 1, 233–240 (1906).

Acree, S. F. On the detection of formaldehyde in milk. *J. Biol. Chem.*, 2, 145–148 (1906–07).

Mottram, V. H. A note on the Hopkins and Cole modification of the Adamkiewicz test for protein. *Biochem. J.*, 7, 249–259 (1913).

Gortner, R. A., and Holm, G. E. On the origin of the humin formed by the acid hydrolysis of proteins. II. Hydrolysis in the presence of formaldehyde. III. Hydrolysis in the presence of aldehydes. *J. Am. Chem. Soc.*, 39, 2447–2501 (1917).

Fearon, W. B. A study of some biological tests. No. 2. The Adamkiewicz protein reaction. The mechanism of the Hopkins-Cole test for tryptophan. A new colour test for glyoxylic acid. *Biochem. J.*, 14, 548–564 (1920).

Expt. 82. ***p*-Dimethylaminobenzaldehyde Test.**—To 15 cc. of dilute hydrochloric acid (1 : 1), add a few milligrams of powdered fibrin, and 1 cc. of *p*-dimethylaminobenzaldehyde reagent. Mix thoroughly and incubate at 37° C. for 24 hours. A blue color develops. This depends upon the fact that the aldehyde condenses with the α-position of the indole nucleus of tryptophane. Since the intensity of the color is governed by the amount of tryptophane present, this is the best qualitative test for indicating the relative amount in individual proteins. Also perform the test using casein, gelatin, Witte's peptone, silk peptone (Expt. 100), Bacto-peptone, and proteose-peptone,[1] and compare the six results. Have the peptones been formed by acid or enzyme hydrolysis of proteins?

p-Dimethylaminobenzaldehyde reagent.—Prepare a 5 per cent solution of the aldehyde in 10 per cent sulfuric acid.

Rohde, E. Die Farbenreaktionen der Eiweisskörper mit *p*-Dimethylaminobenzaldehyde und anderen aromatischen Aldehyden. *Z. physiol. Chem.*, 44, 161–170 (1905).

Herzfeld, E. Über eine quantitative Tryptophanbestimmungsmethode. *Biochem. Z.*, 56, 258–266 (1913).

Holm, G. E., and Gortner, R. A. On the origin of the humin formed by the acid hydrolysis of proteins. IV. Hydrolysis in the presence of aldehydes. III. Comparative hydrolysis of fibrin and gelatin in the presence of various aldehydes. *J. Am. Chem. Soc.*, 42, 632–640. Cf. 636 (1920).

*May, C. E., and Rose, E. R. The tryptophane content of some proteins. *J. Biol. Chem.*, 54, 213–216 (1922).

Holm, G. E., and Greenbank, G. R. The quantitative determination of tryptophan. *J. Am. Chem. Soc.*, 45, 1788–1792 (1923).

Burr, G. O., and Gortner, R. A. Humin formed by the acid hydrolysis of proteins. VIII. The condensation of indole derivatives with aldehydes. *J. Am. Chem. Soc.*, 46, 1224–1246 (1924).

[1] This material may be obtained from the Digestive Ferments Company, Detroit, Michigan.

Jones, D. B., Gersdorff, C. E. F., and Moeller, O. The tryptophane and cystine content of various proteins. *J. Biol. Chem.*, 62, 183–195 (1924).

Kraus, Ida. Colorimetric determination of tryptophane by the vanillin-HCl reaction and the quantitative separation from indole and skatole. *J. Biol. Chem.*, 63, 157–178 (1925).

Fellers, C. R., and Clough, R. W. Indole and skatol determination in bacterial cultures. *J. Bact.*, 10, 105–133 (1925).

Expt. 83. Molisch's Test.—To 3–4 cc. of the protein solution in a test tube, add a few drops of a fresh 10 per cent solution of α-naphthol in 95 per cent ethyl alcohol or in chloroform, and mix thoroughly. Pour about 5 cc. of concentrated sulfuric acid down the side of the inclined tube so that the acid forms a layer beneath the protein solution without mixing with it. A purple-red ring develops at the zone of contact. A positive reaction is an indication of the presence of a carboyhydrate group in the protein molecule or in the solution. In the presence of sulfuric acid, the α-naphthol condenses with the aldehyde formed from the carbohydrate by the action of the acid, and makes the colored compound. What is the aldehyde formed from the carbohydrate used?

This test and the two preceding depend on the addition of an aldehyde or of some compound which will react with the aldehyde formed from the protein. In Experiments 81 and 82, the added aldehyde reacts with the specific amino acid present in the protein molecule, and a color is produced. In the Molisch test, the aldehyde is formed from the carbohydrate group present in the protein and then a substance is added which, combined with it, produces a color. There are proteins, such as fibrin, which contain the specific amino acid and which also yield the essential aldehyde. The Liebermann test (Expt. 84) depends upon this type.

Osborne, T. B., and Campbell, G. F. The protein constituents of egg white. *Conn. Agr. Expt. Sta., Report*, pp. 348–375. Cf. 368–372 (1899); reprinted in *J. Am. Chem. Soc.*, 22, 422–450. Cf. 443–447 (1900).

*Mulliken, S. P. A method for the identification of pure organic compounds. Vol. 1, p. 26. John Wiley & Sons, New York, 1905.

Izumi, S. Über die Kohlenhydratgruppe der Glykoproteide. *Z. Physiol. Chem.*, 142, 175–188 (1925).

Expt. 84. Humin Formation.—During acid hydrolysis of proteins, a black insoluble substance, known as *humin*, is formed. This is due to the reaction, resulting from the tryptophane present in the protein molecule and an unknown aldehydic constituent, which may be native to the protein or may be added in its preparation. It is really a reaction between the α-position of the indole nucleus of tryptophane and the aldehyde.

These conclusions on the formation of humin may be illustrated by the following tests with indole or tryptophane. Dissolve 6 mg. of either substance in 3 cc. of 20 per cent hydrochloric acid. Perform each test in a test tube, 100 × 12 mm., using 0.5 cc. of the solution, which is equivalent to 1 mg. of the substance.

(*a*) Add one drop of dilute formaldehyde solution (1 cc. of 37–40 per cent formaldehyde in 20 cc. of distilled water) and heat. The solution becomes yellow and then a yellow precipitate forms; this turns dark brown or black if allowed to stand.

(*b*) Add two or three drops of *p*-dimethylaminobenzaldehyde reagent (Expt. 82) and warm gently. A blue color develops on standing.

(*c*) Add a small quantity of glucose and heat. A rose color followed by a deep reddish-brown develops. If the solution is allowed to stand, a dark brown precipitate separates.

(*d*) Place 5 cc. of old ether or old 95 per cent ethyl alcohol in a test tube and evaporate until only a film of liquid remains; then add the solution and heat. Observe the changes that take place. What does this experiment indicate with regard to the use of ether and alcohol in the purification of proteins?

A test, depending upon a protein which contains *both* the specific amino acid, tryptophane, and an essential aldehyde, is that by Liebermann. Add pulverized fibrin to 20 per cent hydrochloric acid and heat. A violet color develops, followed eventually by the formation of a black precipitate.

Liebermann, L. Wie hat man die bekannte Reaction auf Eiweiss mit Salzsäure anzustellen und wie lässt sie sich zum Nachweis geringer Mengen Eiweiss im Harne verwenden? *Centr. Med. Wissenschaften*, 25, 321–323 (1887); and Zur Eiweissreaction mit Salzsäure. *Centr. Med. Wissenschaften*, 25, 450 (1887).

Cole, S. W. On certain colour reactions of proteid due to tryptophane. *J. Physiol.*, 30, 311–318 (1903–04).

Gortner, Ross Aiken. The misuse of the term "melanin." *Science*, 36, 52–53 (1912).

Burr, G. O., and Gortner, R. A. The humin formed by the acid hydrolysis of proteins. VIII. The condensation of indole derivatives with aldehydes. *J. Am. Chem. Soc.*, 46, 1224–1246 (1924).

Expt. 85. Ehrlich's Diazo Test.—Place 5 cc. of 1.1 per cent anhydrous sodium carbonate solution in a test tube, add 2 cc. of the reagent from a pipet, and mix the contents of the test tube thoroughly. This should not take more than 30 seconds. One minute after the reagent begins to mix with the sodium carbonate, add 1 cc. of the protein solution. Again mix the contents of the test tube *thoroughly*. Observe the gradual development of color which occurs in from 3 to 10 minutes. A yellow color, which changes rapidly to orange and then

more slowly to pink, is produced. Perform this test also with a 1 per cent gelatin sol. This method is based upon the interaction between the imidazole ring and *p*-phenyldiazonium sulfonate (*p*-diazobenzene sulfonate). It may be used for the colorimetric estimation of histidine and other imidazole derivatives. Tyrosine also responds to the test.

Stock sulfanilic acid.—Dissolve 0.9 gm. of sulfanilic acid in 9 cc. of hydrochloric acid of sp. gr. 1.19 in a 100 cc. volumetric flask and dilute to the mark with distilled water.

Stock sodium nitrite.—Dissolve 5 gm. of sodium nitrite in distilled water in a 100 cc. volumetric flask and dilute to the mark.

The reagent, p-diazobenzene sulfonic acid solution.—Pipet 1.5 cc. of each of the stock solutions into a 50 cc. volumetric flask. Immerse it in an ice bath for 5 minutes. Add 6 cc. more of the stock sodium nitrite solution, shake well, and allow the flask to lie in the ice bath for 5 minutes. Dilute to the mark with distilled water, mix thoroughly, and return the flask to the ice bath where it should be kept. The reagent must not be used for at least 15 minutes after dilution. Make a fresh reagent each day that it is to be used.

Ehrlich, P. Einige Worte über die Diazoreaction. *Deut. med. Wochschr.*, 9, 549–552 (1883).

Ehrlich, P. Uber die Sulfodiazobenzol-Reaction. *Deut. med. Wochschr.*, 10, 419–422 (1884).

Pauly, H. Uber die Konstitution des Histidins. I. Mitteilung. *Z. physiol. Chem.*, 42, 508–518. Cf. 516–518 (1904).

Totani, G. On the diazo reactions of histidine and tyrosine. *Biochem. J.*, 9, 385–392 (1915).

*Koessler, K. K., and Hanke, M. T. Studies on proteinogenous amines. II. A microchemical colorimetric method for estimating imidazole derivatives. *J. Biol. Chem.*, 39, 497–519 (1919).

Hanke, M. T., and Koessler, K. K. Studies on proteinogenous amines. VII. The quantitative colorimetric estimation of histidine in protein and protein-containing matter. *J. Biol. Chem.*, 43, 527–542 (1920).

IV. Color Reactions of Free Amino Acids

Expt. 86. Piria's Test for Tyrosine.—Place about 1.0 mg. of tyrosine in a small evaporating dish, moisten it with one or two drops of concentrated sulfuric acid, and allow to stand for half an hour. Then add sufficient distilled water to make a final volume of about 4 cc. Neutralize the solution at the boiling temperature with precipitated calcium carbonate and filter. Prepare a *fresh* solution of ferric chloride by dissolving a particle in a small amount of distilled water. Add it to the filtrate, drop by drop, with constant shaking until a reddish-violet

color develops. What color reaction of the proteins also serves as a test for tyrosine?

*Piria, R. Notiz über das Tyrosin. *Ann. Chem. Pharm.*, 82, 251–252 (1852).

Expt. 87. Denigè–Mörner Test for Tyrosine.—To a small quantity of tyrosine add 2–3 cc. of the sulfuric acid-formaldehyde solution and heat to boiling. After a short time a permanent green color develops. If an aqueous solution of tyrosine is used, bring it to a concentration of 55 per cent sulfuric acid by the addition of slightly more than an equal volume of concentrated acid, then add the reagent. Heat the solution to boiling as before and, when the acid has reached the required concentration, the green color appears.

Sulfuric acid-formaldehyde solution.—Mix 1 cc. of 40 per cent formaldehyde, 45 cc. of distilled water, and 55 cc. of concentrated sulfuric acid.

*Denigè, G. Nouvelle réaction coloré de la tyrosine. *Compt. rend.*, 130, 583–585 (1900).

*Mörner, C. T. Kleinere Mittheilungen. *Z. physiol. Chem.*, 37, 86–93. Cf. 86–87 (1902–03).

Expt. 88. Bromine Test for Tryptophane.—To a slightly alkaline solution of tryptophane, add a saturated solution of bromine water, drop by drop, from the end of a stirring rod. Shake the solution after the addition of each drop and allow time for the color to develop. A pink, violet or red color or a red precipitate forms, depending upon the concentration. The latter changes to yellow upon the addition of more of the halogen. The different colors are due to the formation of halogen substitution products. Both tryptophane and tyrosine are among the early products of tryptic digestion. The progress of the hydrolysis of the protein molecule by the enzyme, trypsin, may be followed in this reaction.

Expt. 89. Test for Dihydroxyphenylalanine.—Place 2 gm. of finely ground velvet bean (*Stizolobium deeringianum*) meal and 10 cc. of distilled water in a test tube, and close with a stopper. Extract for about 10 minutes, shaking at intervals, and then filter. Expose a portion of the filtrate to the air and observe the several shades of color through which it passes. To the other portion add a drop of ferric chloride solution; a light green color develops. Next add either sodium acetate, sodium carbonate, or ammonium hydroxide solution, and observe the change to reddish-purple.

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V. Isolation of Natural Plant Proteins

A. simple proteins

Of the seven groups of simple proteins, four are characteristic of plants. These are distinguished from each other by their different solubilities in various solvents. They are as follows: (1) The albumins, which are soluble in water, dilute salt solutions, dilute acids and alkalis, which are insoluble in alcohol at or near their isoelectric points. (2) The globulins, which are soluble in dilute salt solutions, dilute acids and alkalis, but which are insoluble in water and in alcohol at or near their isoelectric points. (3) The prolamines, which are soluble in 60 or 80 per cent ethyl alcohol, dilute acids and alkalis, but relatively insoluble in absolute alcohol, water, and dilute salt solutions. (4) The glutelins, which are soluble in dilute acids and alkalis, but which are practically insoluble in water, dilute salt solutions, and alcohol.

Expt. 90. Albumin from Egg White.—Thoroughly stir the whites of 36 fresh eggs with an equal volume of saturated pure ammonium sulfate solution, and filter off the precipitate of ovoglobulin at once. To the clear reddish-yellow filtrate, contained in a precipitating jar, add saturated ammonium sulfate solution until the slight precipitate, which is indicated by a milky turbidity, ceases to dissolve and becomes permanent. Then add 0.2 *N* sulfuric acid until the mixture has a hydrogen-ion concentration of pH 4.58, the optimum for crystallization. During the addition of the sulfuric acid the precipitate is first dissolved; the color of the solution changes from reddish-yellow to yellow, and finally there is formed an amorphous precipitate, which is at first easy, but later more difficult, to dissolve by stirring. At about pH 4.58, it should be just possible to dissolve this precipitate. Stir the resulting opalescent solution vigorously; crystallization will usually begin in about an hour. Allow the mixture to stand at room temperature from 5 to 6 days, stirring frequently.

Then filter over night on three large gravity funnels; should any mother liquor remain above the precipitate next day, remove it with a pipet. Wash each precipitate twice with an ammonium sulfate solution of such concentration that the precipitation of the filtrate is just avoided.

Fill each filter completely without stirring up the precipitate, and the washing solution will gradually displace the mother liquor. To determine the concentration of the washing solution, prepare a series of test tubes, each containing 10 cc. of saturated ammonium sulfate solution and a different volume of distilled water; to each add a few cubic centimeters of the clear filtrate. When, for example, all the tubes up to and including that containing 6.5 cc. of water show turbidity, while all those containing 7 cc. or more of water are clear, it is evident that the concentration of the washing solution should be in the ratio of 10 cc. of ammonium sulfate solution to 7 cc. of distilled water.

When the second washing solution has filtered over night, remove with a pipet any which has not run through. Scrape the precipitate from the filter papers into a large evaporating dish, using a porcelain spoon. Dissolve the precipitate remaining on the papers with distilled water and add to the evaporating dish; then add, with thorough stirring, just enough more distilled water to dissolve the precipitate completely.

If necessary, filter the solution, and pour it into the precipitating jar; again add saturated ammonium sulfate solution to permanent turbidity, and adjust to a hydrogen-ion concentration of pH 4.58 by the addition of 0.2 *N* sulfuric acid. Stir the solution vigorously, and allow to stand 3 or 4 days.

Filter the crystallized egg albumin, wash with an ammonium sulfate solution of the proper concentration, and then dissolve the precipitate in distilled water. Dialyze (Expt. 3) in a large hardened collodion bag until *free* from sulfates. Early in the dialysis, observe whether the collodion bag has been sufficiently hardened, by testing the dialysate for the presence of albumin according to the method of Sörensen.

The purified egg albumin solution will preserve its characteristic properties for months if mixed with a large quantity of toluene, shaken frequently, and kept at about 0° C.

If it is desirable to use egg albumin in the dry form, heat the dialyzed egg albumin solution to 70° C. in a water bath and filter off the coagulum. Dry in an oven at 70° C. and pulverize. It is not possible to redissolve the pulverized egg albumin in water, hence this is an unsuitable form for experiments requiring egg albumin solution.

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Expt. 91. Globulin. Arachin and Conarachin from Peanuts.—*Preparation of the meal.*—Grind shelled unroasted peanuts (*Arachis hypogaea*) to a fine meal either in a food chopper or in a mill. Using ether or petroleum ether, extract the meal completely, either in a flask at room temperature or in a Schmidt fat extractor as illustrated in Fig. 17. This contains a 5-liter round-bottomed Pyrex flask, *F*, closed with a 4 hole tin foil-covered cork stopper. In this are inserted a dropping funnel, a thermometer, a siphon tube, *H*, constricted at *P*, and an outlet tube, *E*, which leads to the large porcelain jar, *B*. The latter has a mercury trough, *V*, into which the cover, *A*, is set, thus making a vapor-tight joint. The cork stoppers, *M*, *N*, and *O*, are also sealed with mercury. Stopper, *N*, contains a spiral water-cooled condenser. To charge the apparatus, place a layer of cotton in the bottom of *B*, and introduce the ground meal. Fill *F* three-fourths full of the solvent through the dropping

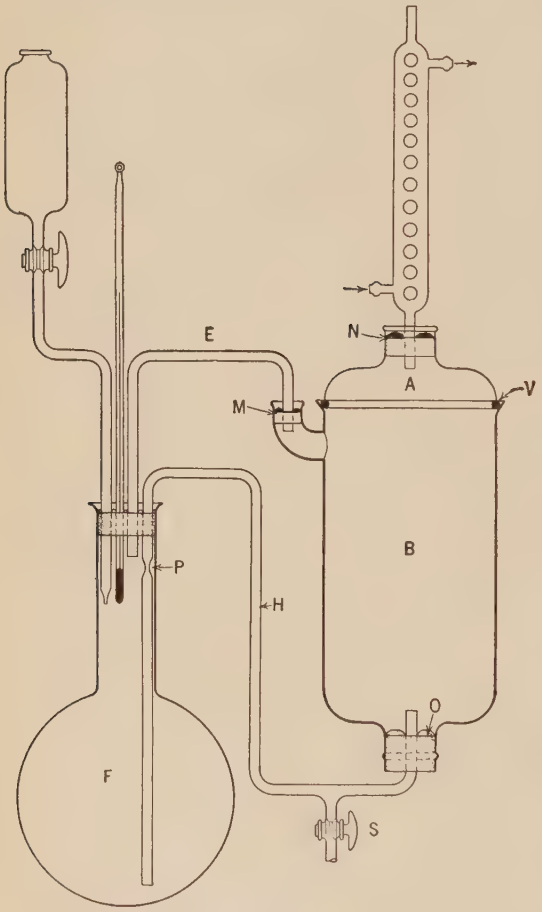

Fig. 17.

funnel and heat gently on a small electric hot plate. When the extraction is complete, the solvent remaining in *B* may be drawn off through the stopcock, *S*. Should the flask method be used, place the meal in a large Erlenmeyer flask; add sufficient ether to cover it, and allow to stand one hour, shaking frequently. Filter off the ether by suction on a Büchner funnel and extract again with fresh ether. Repeat the process until the oil is entirely removed, preserving the extract for the recovery of the ether. Dry the extracted meal where there is no danger from fire, until free from the odor of ether.

Extraction and precipitation of the globulins.—Stir into 1.25 liters of a 10 per cent sodium chloride solution 250 gm. of the extracted air-dried peanut meal; allow to stand several hours, stirring frequently if possible. Using a small portion of the mixture at a time, place it in an 8-oz. duck cloth and squeeze and wring thoroughly with the hands until all of the liquid has been removed. A volume of extract, equal to about 80 per cent of the sodium chloride used, should be obtained. Filter this extract through paper pulp on a Büchner funnel, or pass it through a Sharples laboratory super-centrifuge.

To prepare *paper pulp*, tear filter paper clippings [2] into small pieces; place these in an Erlenmeyer flask containing distilled water; and shake until a fairly thick pulp, which contains no lumps of the original paper, is formed. Fit a filter paper to a 10 cm. Büchner funnel and upon this pour the pulp to form a layer 4–5 mm. thick. The layer of pulp should be thicker if a wider Büchner funnel is used. As the water drains through, the pulp is drawn down more in the center than on the sides, so that the surface of the filter becomes somewhat saucer shaped. Press the pulp down firmly with the fingers and wash it several times with distilled water. Pour on the extract and conduct the filtration with the least possible suction. If the filter becomes clogged by the deposit of fine material, rubbing slightly with a glass rod or spatula will break the surface and allow filtration to proceed. Do this very cautiously, since it often tears the filter and causes loss of time and material by refiltering.

The globulins may be precipitated by saturating the filtrate with carbon dioxide. Dilute the filtrate with two volumes of distilled water and then saturate the mixture by passing through it a stream of carbon dioxide from either a cylinder or a Kipp generator. To the exit tube of the generator attach an absorption tube, which consists of a glass tube, having on one end a bulb perforated with a large number of holes. Occasionally add to the mixture a few drops of a foam inhibitor, such as

[2] These are small clippings left after cutting filter paper circles. They may be obtained from most chemical supply houses.

phenyl ether. Allow it to stand over night so that the white precipitate will settle. Filter on a Büchner funnel or centrifuge.

Isolation of arachin.—Redissolve the residue in 10 per cent sodium chloride solution and filter. To the clear filtrate add gradually, fine powdered ammonium sulfate until the solution becomes 0.2 saturated with the salt. First calculate the amount of ammonium sulfate required at the temperature of the laboratory to produce 0.2 saturation of the volume of clear filtrate used. A coagulum begins to form at 0.15 saturation and this increases rapidly until 0.2 saturation is reached. Allow the mixture to stand over night or longer so that the coagulum may settle. Decant the supernatant liquid and preserve it for the isolation of conarachin. Using a Büchner funnel, wash the coagulum with a 10 per cent sodium chloride solution containing ammonium sulfate to 0.2 saturation. Then dissolve the residue in the least possible quantity of 10 per cent sodium chloride solution and filter. Dialyze the filtrate in a collodion bag (Expt. 1) against distilled water until free from chlorides, according to the continuous process (Expt. 2). Filter off the white precipitate which is arachin, on a Büchner funnel or use a Sharples laboratory super-centrifuge. Wash the arachin twice with 50 per cent ethyl alcohol, once with 95 per cent ethyl alcohol and finally with ether. Dry at room temperature in a place free from dust.

Isolation of conarachin.—Completely saturate the filtrate obtained from the isolation of arachin with finely powdered ammonium sulfate. Allow this to stand over night or longer, filter, and wash with a saturated ammonium sulfate solution. Dissolve the residue in a 10 per cent sodium chloride solution, filter, and dialyze the filtrate in a collodion bag against distilled water until free from chlorides. Filter, wash, and dry the precipitate, which is conarachin, according to the method described for arachin.

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Expt. 92. Globulin. Edestin from Hemp Seed.—*Preparation of the meal.*—Completely extract freshly ground hemp seed (*Cannabis sativa* L.) meal, using ether or petroleum ether, in either a Schmidt fat extractor (Expt. 91) or a flask at room temperature. For the latter method, place the meal in a large Erlenmeyer flask; add sufficient ether to cover it, and allow to stand one hour, shaking frequently. Filter off the ether by suction on a Büchner funnel and extract again with fresh ether. Repeat the process until the oil is entirely removed, preserving the extract for the recovery of the ether. Dry the extracted meal where there is no danger from fire, until free from the odor of ether.

Method. I. *Extraction with sodium chloride.*—Heat 750 cc. of a 10 per cent sodium chloride solution to 65° C. and, while stirring, add 250 gm. of the extracted air-dried hemp seed meal; allow to stand one hour, stirring frequently. Again warm the mixture to 65° C. by placing the container in a water bath heated to about 75° C.; stir frequently during the process. Moisten an 8-oz. cotton duck cloth with a hot 10 per cent sodium chloride solution, and, placing in it a portion of the mixture at a time, squeeze and wring thoroughly with the hands until all of the liquid has been removed. A volume of extract, equal to about 80 per cent of the sodium chloride solution used, should be obtained. Heat this extract to 65° C. and filter it into an Erlenmeyer flask through a hot water jacketed funnel at 70° C. Close the flask with a stopper; cool to room temperature; then place in a refrigerator over night. Filter off the crystals on a Büchner funnel, fitted with a hardened filter paper, keeping them covered with the liquid until it has all been transferred. Return the edestin to the same Erlenmeyer flask and dissolve it in 10 per cent sodium chloride solution, heated to 60–65° C. as before. If, because of the presence of edestan (Expt. 97), the solution appears turbid, filter it through a hot water jacketed funnel at 70° C.; cool to room temperature and place in the refrigerator for 2 or 3 hours. Filter the crystallized edestin on a Büchner funnel as before. Edestin may also be obtained by dialysis of the filtrate. Wash the crystals twice with 50 per cent ethyl alcohol, twice with 95 per cent ethyl alcohol, twice with absolute ethyl alcohol, and finally with ether. Dry in the air, protected from dust, or at 35–40° C. The edestin usually crystallizes in simple octahedrons, although modifications of this form are frequently observed. Examine some of the crystals under the microscope.

Method II. *Extraction with sodium benzoate.*—Mix 250 gm. of the extracted air-dried hemp seed meal with about 350 cc. of 0.5 *N* sodium benzoate solution; allow to stand an hour, stirring frequently. Press out the extract through an 8-oz. cotton duck cloth in a hydraulic press.

Again extract the residue with a fresh portion of sodium benzoate equal to the volume of liquid extracted. Allow the mixture to stand half an hour and again press. Filter the combined extracts through paper pulp (Expt. 91) on a Büchner funnel, or pass it through a Sharples laboratory super-centrifuge. Pour the filtrate into about 10 liters of distilled water and stir thoroughly. The precipitation of some proteins may be brought about by *dilution*, the relative mass of the protein and salt being thus unaltered. Allow the precipitate to settle, decant the clear supernatant liquid and wash the precipitate with distilled water by decantation three or four times. Dialyze (Expt. 2) the suspended precipitate for about 48 hours or until free from sodium benzoate. Filter the crystals on a Büchner funnel, wash, and dry as described in Method I.

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Expt. 93. Prolamine. Gliadin from Wheat Flour.—Place 350 gm. of wheat (*Triticum vulgare*) flour [3] in a suitable container and while stirring, gradually add distilled water until a stiff dough is formed. This will require about 60 cc. of water for each 100 gm. of flour. Knead the dough thoroughly and allow it to remain under water for at least half an hour. Then wash out the starch by kneading it in a stream of water. From 115–125 gm. of crude moist gluten should be obtained;

[3] Gluten can be washed from a "strong" wheat flour much more readily than from a "weak" flour (Expt. 45, footnote).

of this about 65 per cent is water. Cut or tear the gluten into *very small* pieces and place in a flask; add sufficient 95 per cent ethyl alcohol and distilled water to form, with the water in the gluten, 600 cc. of 70 per cent ethyl alcohol by volume (Expt. 3, footnote). Heat the flask in a water bath at 70° C. under a reflux condenser for an hour and a half, shaking at frequent intervals. Filter or centrifuge the extract and concentrate the clear filtrate to a thick sirup in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) until it is impossible to prevent froth from getting into the alcoholic distillate. Use this alcoholic distillate for a second and third extraction of the gluten in the manner just described. Preserve the gluten residue for the preparation of glutenin (Expt. 94). Cool the sirup in the Claisen flask, and then pour it slowly, with constant stirring, into a large volume of ice-cold distilled water, containing 10 gm. of sodium chloride per liter; the gliadin separates as a gummy mass. Wash it several times with distilled water; then dissolve in 70 per cent ethyl alcohol and filter. If it is desirable to prepare absolutely pure gliadin, concentrate the clear alcoholic filtrate to a sirup under diminished pressure, reprecipitate by pouring into distilled water containing 1 per cent of sodium chloride, redissolve in 70 per cent ethyl alcohol and filter; carry out this process several times. Finally pour the sirup into a mixture of absolute ethyl alcohol and ether. The gliadin separates in flocculent form. It may be necessary to add a small amount of sodium chloride to the solution to bring about complete separation. Filter the gliadin and wash with absolute ethyl alcohol and then with ether. Dry in a vacuum desiccator over sulfuric acid. The gliadin thus prepared contains a little sodium chloride.

Instead of removing the alcohol from the third extraction by concentrating under diminished pressure and finally precipitating the gliadin, the same result may be obtained by dialysis. For this purpose, mix the third alcoholic extract with the sirup remaining from the two previous concentrations and dialyze (Expt. 2) in a collodion bag against distilled water. The gliadin separates in flocculent form as the alcohol is displaced by the water; it may be removed and washed as directed above for pure gliadin.

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Expt. 94. Glutelin. (*a*) *Glutenin from wheat flour.*—Dry the gluten residue, remaining from the alcoholic extraction of gliadin (Expt. 93), at room temperature; pulverize, and then thoroughly extract with ether to remove fat; again dry at room temperature. Shake the powder with sufficient 0.2 per cent potassium hydroxide solution to disperse it and filter. Neutralize the clear filtrate with very dilute hydrochloric acid; the glutenin separates in flocculent form. Allow to settle, decant the supernatant liquid, wash several times with distilled water by decantation, then with 70 per cent ethyl alcohol and last with 95 per cent ethyl alcohol. Finally dehydrate the glutenin thoroughly with absolute ethyl alcohol, wash with ether and dry in a vacuum desiccator over sulfuric acid. The glutenin thus prepared contains a small amount of potassium chloride.

To prepare absolutely gliadin-free glutenin, it is necessary to extract the original flocculent mass repeatedly with 70 per cent ethyl alcohol; dry and pulverize the residue; redissolve in 0.2 per cent potassium hydroxide solution; reprecipitate with dilute hydrochloric acid and wash. This purification should be repeated several times.

(*b*) *Quantitative determination of glutenin in wheat flour.*—All the flour proteins are first dissolved in a solution which is approximately 70 per cent by volume with respect to methyl alcohol and less than 0.025 *N* with respect to sodium hydroxide. When the starch is removed by filtration, the glutenin is quantitatively precipitated at its iso-electric point, the other proteins remaining in solution.

Place 8 gm. of wheat flour in a dry 200 cc. Kohlrausch sugar flask or other extra capacity 200 cc. flask; add 50 cc. of distilled water and mix thoroughly; then add from a pipet 5 cc. of *N* sodium hydroxide solution, rotating the flask meantime. Thereafter shake thoroughly at intervals of 10 minutes during an hour. At the end of this time, add acetone-free methyl alcohol in 50 cc. portions until the 200 cc. mark is

reached; shake the flask after each addition. To compensate for the volume occupied by the flour, add an excess of 5 cc. of methyl alcohol. The starch settles quickly, leaving a clear supernatant liquid which contains the proteins. Filter through a filter paper on an ordinary funnel. Pipet 50 cc. of the filtrate, which contains the equivalent of 2 gm. of flour, into a 100 cc. Erlenmeyer flask and add a few drops of brom thymol blue (Expt. 72). To precipitate the glutenin add, while shaking the flask, 0.2 *N* hydrochloric acid from a buret until a light olive color, corresponding to a pH of about 6.4, results. However, precipitation seems to be complete anywhere between pH 6.0 and 6.8. Allow the glutenin to settle for an hour when the supernatant liquid should be clear. Pour the contents of the flask into a 100 cc. centrifuge tube and centrifuge for 10 minutes. This operation may be delayed an hour or two. Completely decant the clear supernatant liquid and then loosen the mass of glutenin from the centrifuge tube by the addition of a small quantity of distilled water. Pour into a Kjeldahl flask and determine the nitrogen (Expt. 113*a*); multiply the result by the conversion factor 5.7, to obtain the amount of glutenin.

This method may also be applied in the rapid preparation of pure glutenin. In this case, use 80 gm. of wheat flour and carry out all operations as described above. Wash the precipitate several times by decantation with 70 per cent methyl alcohol at a pH of 6.4 as indicated by brom thymol blue.

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B. CONJUGATED PROTEINS

Expt. 95. Nucleoprotein. Nucleic Acid from Brewer's Yeast.—This method for the preparation of nucleic acid is based on the following facts: first, that nucleic acid exists in yeast as a nucleoprotein, that the latter is a type of acid protein and that, when treated with dilute alkali at room temperature, hydrolysis takes place with the formation of the alkali salt of nucleic acid and protein; second, that sodium nucleate is soluble in dilute acetic acid; and third, that nucleic acid is precipitated as a mixture of the mono- and di-magnesium salts by magnesium sulfate and hydrochloric acid.

Dilute about 15 kg. (5 gallons) of fresh brewer's yeast (*Saccharomyces cerevisiae*) with 2 parts of tap water and add, with constant stirring, a concentrated solution of commercial sodium hydroxide, using 50 gm. for each kilogram of yeast. Stir this mixture vigorously from 8 to 10 minutes and then treat immediately with enough concentrated hydrochloric acid to neutralize about 85 per cent of the sodium hydroxide used. Next neutralize the remainder of the alkali with 30 per cent acetic acid; then carefully add enough more acid to bring about complete precipitation, but not enough to cause the precipitate to dissolve. At this point the mixture is distinctly acid to litmus paper. The alkaline solution of yeast is brown while the acid solution is grayish-white. Allow the protein precipitate to settle over night. Siphon off the supernatant liquid and pass it through a Sharples laboratory super-centrifuge, or filter through paper pulp (Expt. 91) on a Büchner funnel. If the liquid foams during centrifuging, add a foam inhibitor, such as ether.

To the slightly opalescent filtrate add sufficient finely pulverized magnesium sulfate to make a 4 per cent solution. Take 500 cc. and add hydrochloric acid (1 : 1) from a buret, with stirring, until precipitation is just complete. From time to time during the addition, stop stirring and allow the mixture to become quiet so that any flocking out of the nucleic acid may be observed. *Exercise great care* that there may not be an *excess* of hydrochloric acid, or it will redissolve the nucleic acid producing a milky solution. From the amount of acid needed to precipitate this portion of the solution, calculate the amount of concentrated hydrochloric acid necessary to precipitate the entire solution and add it slowly with constant stirring. Reserve the last 25–40 cc. of hydrochloric acid and add only when assured that precipitation is not complete. The nucleic acid agglutinates quickly, forming large flocks which settle in a short time. Siphon off the supernatant liquid and discard it. To the precipitate add an equal volume of 60 per cent ethyl alcohol, mix thoroughly, allow to settle, and decant. Again wash successively with 80 per cent and 95 per cent alcohol; filter on a Büchner funnel, fitted with a hardened filter paper, and drain as dry as possible, finally using the rubber pressure device (Expt. 101). Remove the residue to a mortar and grind it with sufficient absolute ethyl alcohol to form a thin paste; again filter as before, using the same filter paper. Transfer to the mortar, grind with ether, and filter. Dry quickly at 45–50° C. or in a vacuum desiccator over sulfuric acid. If dried too slowly the substance will hydrate and become brown.

If desired, the salt of nucleic acid may be further purified as follows: dissolve it in 0.1–0.2 per cent sodium hydroxide; add 30 per cent acetic acid until no more precipitate forms, but do not let the precipitated

impurities dissolve. The nucleic acid must remain in solution during this process. Filter the solution and treat it with magnesium sulfate and hydrochloric acid as previously described. When large quantities of yeast are utilized, a very pure product is obtained after one purification. Pure nucleic acid should not respond to the biuret test (Expt. 76). Try it. What does this indicate? Do plant and animal nucleic acids differ in chemical constitution?

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Levene, P. A., and Sobotka, R. The thio-sugar from yeast. *J. Biol. Chem.*, 65, 551–554 (1925).

Jones, W., and Perkins, M. E. The nitrogenous groups of plant nucleic acids. *J. Biol. Chem.*, 62, 557–564 (1924–25).

Expt. 96. Guanine and Adenine from Yeast Nucleic Acid.—Place 25 gm. of yeast nucleic acid (Expt. 95) in a flask and add 500 cc. of 10 per cent sulfuric acid; connect the flask with a reflux condenser, and immerse it in a boiling water bath for 2 hours. While still hot, treat the pale yellow solution with concentrated ammonium hydroxide until the approximate neutral point is reached, and then add an excess, so that the solution will contain about 2 per cent of ammonium hydroxide. Add the reagent slowly after the precipitation of guanine begins. It will separate in granular form while the adenine remains in solution. Allow to cool and filter, saving the filtrate for the subsequent preparation of adenine. Wash the guanine with 1 per cent ammonium hydroxide solution and add the washings to the original filtrate. Suspend the guanine in boiling water and dissolve it by the addition of the least possible amount of 20 per cent sulfuric acid. Decolorize by adding a few grams of the vegetable decolorizing carbon, Norit, and filter. Heat the solution to the boiling point, and reprecipitate the guanine by the addition of an excess of ammonium hydroxide solution as before. Again cool, filter, wash with 1 per cent ammonium hydroxide solution, and dry at 40° C. Add this filtrate and washings to the original filtrate and washings containing the adenine. Dissolve the guanine in 20 to 25 times its weight of boiling 5 per cent hydrochloric acid and allow to cool. Slender prisms or needles of guanine chloride,

$$C_5H_5N_5O \cdot HCl \cdot 2H_2O,$$

separate. Filter the solution, wash the crystals with very dilute hydrochloric acid, and dry in the *air*.

Using the ammoniacal filtrate and washings containing the adenine, remove by filtration any traces of guanine which may have separated on standing. Slightly acidify the filtrate with 20 per cent sulfuric acid; add 10 per cent copper sulfate solution, and boil vigorously for several minutes. Filter while hot and wash thoroughly with boiling distilled water. The adenine is precipitated as an adenine-cuprous compound. When a boiling solution, containing the purine bases and an excess of copper sulfate, is treated with a reducing substance, such as sodium bisulfite, there is formed a white precipitate of purine-cuprous compound

of the type $R \cdot Cu_2O$, which is practically insoluble in boiling water. As long as the copper sulfate is in excess, and purine bases are present, the continued addition of sodium bisulfite increases the white precipitate; but when the purine bases have been completely precipitated, the further addition of bisulfite and the boiling produce cuprous oxide and the white precipitate takes on a yellowish cast. The addition of the bisulfite is then discontinued and the mixture is boiled vigorously. Since the adenine solution in this experiment contains the reducing sugar, *d*-ribose, it is not necessary to add sodium bisulfite.

Now suspend the adenine-cuprous compound in hot distilled water, and then decompose it by passing a stream of pure hydrogen sulfide through the hot solution, taking care not to add enough to peptize the precipitated cuprous sulfide; filter and wash. If necessary, decolorize the filtrate with the vegetable decolorizing carbon, Norit. Evaporate the solution to dryness on the water bath and dissolve the residue in the smallest possible amount of hot 5 per cent sulfuric acid; allow to cool. Adenine sulfate $(C_5H_5N_5)_2 \cdot H_2SO_4 \cdot 2H_2O$, separates in crystalline form. Filter, wash with *cold* distilled water, and dry.

*Jones, W. Nucleic acids: their chemical properties and physiological conduct. pp. 107–109. Longmans, Green and Company, London, 1920.

Vickery, H. B., and Leavenworth, C. S. Some nitrogenous constituents of the juice of the alfalfa plant. III. Adenine in alfalfa. *J. Biol. Chem.*, 63, 579–583 (1925).

VI. Preparation of Derived Proteins

Expt. 97. Protean. Edestan from Edestin.—Proteans are produced from soluble proteins by the action of water or very dilute acids. Edestin combines with definite quantities of acid to form salts in which the edestin molecule is unchanged. In the presence of an excess of acid, edestin is converted into edestan. The hydrogen ion is the active agent in the transformation, and the edestin formed is proportional to the concentration of the hydrogen ion and to the time of its action. Suspend 3–5 gm. of crystallized edestin (Expt. 92) in distilled water and add carefully 0.2 per cent hydrochloric acid until it just dissolved; then add several cubic centimeters in excess. Allow the acid solution to stand 2 hours or more, shaking frequently. Neutralize the solution with 0.2 per cent sodium hydroxide using phenolphthalein as an indicator. Filter off the precipitate formed, wash with distilled water and finally with absolute ethyl alcohol. Test its solubility in 10 per cent sodium chloride solution and in 0.2 per cent solutions of hydrochloric acid and sodium hydroxide. How does its solubility in these solvents

compare with that of edestin? What are derived or denaturized proteins? The percentage composition of edestan is practically the same as that of the edestin from which it is derived.

*Osborne, T. B. Ein hydrolytisches Derivat des Globulins Edestin und sein Verhältniss zu Weyl's Albuminat und zur Histongruppe. *Z. physiol. Chem.*, 33, 225–239 (1901); reprinted in *J. Am. Chem. Soc.*, 24, 28–39 (1902).

Wu, H., and Yen, Daisy. Studies of denaturation of proteins. I. Some new observations concerning the effects of dilute acids and alkalies on proteins. *J. Biochemistry* (Japan), 4, 345–384. Cf. 351 (1924–25).

Expt. 98. Metaprotein. Protalbinic and Lysalbinic Acids from Egg Albumin.—Place 500 cc. of distilled water in a 1000 cc. Erlenmeyer flask and dissolve in it 15 gm. of sodium hydroxide; add to the cooled solution 100 gm. of powdered egg albumin, shaking the flask vigorously to prevent the formation of lumps. Then heat the mixture on an actively boiling water bath under a *hood* for about an hour, or until practically all of the particles have dissolved. A large amount of ammonia is evolved during the heating. Filter the solution and carefully add to the filtrate 10 per cent sulfuric acid as long as a precipitate forms. Since hydrogen sulfide is evolved at this stage the solution should be acidified under the hood. Allow to stand 12 hours or more; filter off the precipitate, and wash it with a small quantity of distilled water. The filtrate from the protalbinic acid contains the lysalbinic acid and can be purified if desired. Grind the precipitate to a paste with distilled water and dialyze in a collodion bag against distilled water according to the continuous process (Expt. 2) until the dialysate no longer tests acid. Filter off the precipitate in the dialyzer, and dry in a vacuum oven. The yield of protalbinic acid is about 35 per cent of the weight of egg albumin used.

*Paal, C. Über die Einwirkung, ätzender Alkalien auf Eialbumen. *Ber.*, 35, 2195–2206 (1902).

*Kennedy, Cornelia, and Gortner, R. A. The nitrogen distribution in protalbinic and lysalbinic acids. *J. Biol. Chem.*, 39, 2734–2736 (1917).

Expt. 99. Proteoses and Peptones.—Proteoses and peptones are prepared either by digesting the protein with pepsin in a hydrochloric acid solution, or by hydrolysis with mineral acids. The former method is preferable. In the present state of our knowledge regarding these substances, it is impossible to designate the exact character of a proteose or to indicate the exact point at which the proteose characteristic ends and the peptone begins, and the peptone characteristic ends and the peptide begins. At present the best method for differentiating the various proteoses and peptones is by means of their varying solubilities, especially in different strengths of ammonium sulfate.

For this differentiation, Witte's peptone, which is prepared from fibrin and consists of a mixture of the proteoses and peptones, the former predominating, is most satisfactory. Add 5 gm. of Witte's peptone to 100 cc. of distilled water, and warm gently to dissolve it; just acidify with acetic acid and filter. The filtrate contains all the proteoses and peptones. To separate the primary from the secondary proteoses and the peptones, half saturate the filtrate with ammonium sulfate by adding an equal volume of saturated ammonium sulfate solution. Stir the mixture rapidly with a rubber-tipped stirring rod; the coagulum collects as a gummy mass on the end of the rod. Filter and preserve the filtrate, which contains the secondary proteoses and the peptones. The coagulum consists of a mixture of the primary proteoses, proto- and hetero-proteoses.

Primary proteoses.—Dissolve the coagulum in a small quantity of warm distilled water and use portions of the solution for the following tests:

(*a*) Add a few drops of concentrated nitric acid. A precipitate, which disappears on warming but reappears in cooling, is formed.

(*b*) Add a few drops of copper sulfate solution (Expt. 118).

(*c*) Slightly acidify with acetic acid and add a few drops of freshly prepared potassium ferrocyanide solution.

(*d*) Add a saturated solution of picric acid until a permanent precipitate forms. Heat until it dissolves, and then allow to cool.

(*e*) Slightly acidify with hydrochloric acid and add a few drops of Mayer's reagent (Expt. 59).

(*f*) Add a few drops of phosphotungstic acid solution.

(*g*) Perform the biuret test (Expt. 76). How does the color formed compare with that produced by a pure protein?

Secondary proteoses.—To the solution containing the secondary proteoses and the peptones add, with stirring, sufficient finely pulverized ammonium sulfate to saturate the solution. A coagulum which consists of the secondary or deutero-proteoses forms. This generally sticks to the stirring rod or sides of the beaker. Filter and discard the filtrate which contains a small amount of peptones. Dissolve the coagulum in a small quantity of distilled water and use portions of the solution for the tests, which are given under primary proteoses. Observe and compare each result with that obtained under primary proteoses. Note that in the biuret test (*g*) the presence of considerable ammonium sulfate interferes with the test, so that it is necessary to add concentrated sodium hydroxide solution until the salt has been decomposed.

Peptones.—Dissolve 2.5 gm. of Bacto-Peptone,[4] which is practically

[4] This material may be obtained from the Digestive Ferments Company, Detroit Mich.

free from proteoses, in 50 cc. of distilled water and use portions of the solution for the tests which are given under primary proteoses. Observe and compare the results with those obtained under primary and secondary proteoses. Also use a portion of the solution for the following test:

(*h*) To 1 cc. of peptone solution, add 1 cc. of 2 *N* sodium hydroxide solution and 5 cc. of tannic acid solution, and dilute to 20 cc. with distilled water. Shake the mixture throughly and allow to stand 1 hour at 20° C.

Tannic acid solution.—Dissolve 20 gm. of sodium sulfate and 20 gm. of tannic acid in 100 cc. of 0.1 *N* sulfuric acid.

*Pick, E. P. Untersuchungen über die Proteinstoffe. II. Ein neues Verfahren zur Trennung von Albumosen und Peptonen. *Z. physiol. Chem.*, 24, 246–275 (1898).

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*Pick, E. P. Zur Kenntniss der peptischen Spaltungsprodukte des Fibrins. 1. Theil. *Z. physiol. Chem.*, 28, 219–287 (1899).

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*Haslam, H. C. Separation of proteins. Part II. Deutero-albumose. *J. Physiol.*, 36, 164–176 (1907–08).

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*Wasteneys, H., and Borsook, H. A method for the fractional analysis of incomplete protein hydrolysates. *J. Biol. Chem.*, 62, 1–14 (1924).

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Rudd, G. V. Proteoses, peptones, and polypeptides. *Australian J. Exptl. Biol. Med. Sci.*, 1, 187–190 (1924).

Expt. 100. Silk Peptone or Peptone "Roche."—Dry 100 gm. of silk waste [5] for 48 hours at 100° C. Digest the dry material with from three to four times its weight of 70 per cent sulfuric acid for 4 days at ordinary temperature. Note the exact amount of sulfuric acid used.

[5] This material can be supplied by Cheney Brothers, South Manchester, Connecticut.

Pour the chocolate brown peptone solution into ten times its volume of distilled water, contained in a vessel cooled with an ice mixture. Precipitate the sulfuric acid by the addition of the calculated amount of finely pulverized barium hydroxide; stir occasionally, or if possible, use a motor-driven stirrer. Allow the mixture to stand about 12 hours, then filter off the barium sulfate through a double filter paper on a Büchner funnel. Either grind the barium sulfate in a ball mill with about 1000 cc. of distilled water for an hour, or grind it repeatedly in a mortar with distilled water, and filter as above. Use water at 25° C. to extract the barium sulfate when the sulfuric acid has not been completely neutralized with barium hydroxide, that is, when an excess of either sulfuric acid or barium hydroxide is present. If *hot* water is used, there is a further splitting of the peptone to form amino acids. After the combined filtrates from the barium sulfate are free from sulfuric acid and barium hydroxide, concentrate them in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136). The distillation generally proceeds very smoothly, but sometimes vigorous foaming hinders the concentration of the solution. In such case either allow the silk peptone solution to drop into the Claisen flask from a dropping funnel during distillation or place the solution in the Claisen flask and add a few drops of a foam inhibitor, such as paraffin oil or phenyl ether, occasionally. When the peptone solution has been concentrated to 400–500 cc., test again for sulfuric acid and barium hydroxide. If an excess of barium hydroxide is present, evaporate 2–5 cc. of the peptone solution in a weighed platinum or silica dish and ignite the residue. If the ash contains barium oxide, warm the solution to 60° C., then add the calculated quantity of a standard solution of sulfuric acid, and filter. Ordinarily this removes the last trace of barium hydroxide. Further concentrate the peptone solution to about 125 cc. Pour the yellowish-brown solution, with constant stirring, into a *definite* volume of absolute ethyl alcohol. Continue the addition of the solution *only* as long as it separates as a colorless flocculent precipitate. If too much of the silk peptone solution is added, the precipitate becomes slightly sticky. At once discontinue its addition and repeat the operation, using fresh alcohol and observing the same precautions as before. At least 2 liters of absolute ethyl alcohol will be required for the experiment. One hundred grams of silk waste yields 20–30 gm. of silk peptone. This may be used in tests for the activity of erepsin (Expt. 194).

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Mitchell, H. H., and Eckstein, H. C. A foam inhibitor in the Van Slyke amino nitrogen method. *J. Biol. Chem.*, 33, 373–375 (1918).

VII. Preparation of Amino Acids

In the preparation of amino acids, distillation under diminished pressure or *in vacuo* is frequently necessary; this is accomplished by an apparatus set up as shown in Fig. 18. It consists essentially of two parts, three Pyrex flasks fastened together with rubber stoppers, and a manometer. The first part includes a 1000 cc. Claisen distilling flask, which is used for the distillation; a 1000 cc. ordinary distilling flask, which is cooled by a stream of running water and serves as a condensing and receiving flask; and a 200 cc. distilling flask, which is a guard flask. However, it is not necessary to attach the latter except for the determination of ammonia nitrogen (Expt. 113 *d*). The outlet tube of the Claisen flask projects into the bulb of the receiving flask so that the vapors of the distillate will not be drawn off by suction. An ordinary glass tube drawn out to a fine capillary is inserted through the rubber stopper of the Claisen flask, extending almost to the bottom. A piece of rubber tubing, having either a glass stop-cock or a screw clamp to regulate the air supply, is attached to the open end of the tube. This arrangement insures continuous boiling without *bumping*, the vapor phase being produced by passing a slow continuous stream of air through the liquid. The side arm of the Claisen flask is closed with a rubber stopper carrying a glass stop-cock. The outlet tube of either the guard flask or the receiving flask is connected through an Erlenmeyer filtering flask with a good water filter pump and with the manometer, by means of a T tube and rubber pressure tubing. The vacuum obtained from a pump of this type depends upon the pressure and the vapor tension of the water; the latter in turn depends upon the temperature. The tube which connects the filtering flask with the pump should extend to the bottom of the flask, so that any water which runs back because of unequal pressure in the water main will be sucked out as soon as the pressure returns. Various check valves have also been devised to overcome this difficulty; among them are those recommended by Brewster, and Langley.

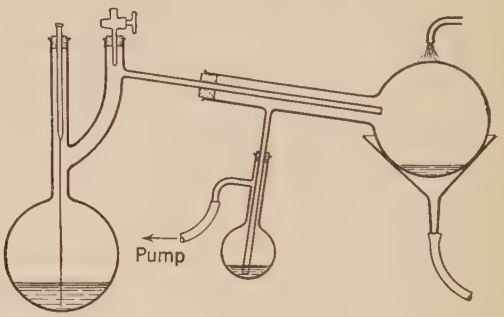

Fig. 18.

The manometer consists of a bent barometer glass tube mounted on a background, one end extending nearly to the bottom of a small bottle containing mercury, the other connected through a glass stop-cock with the T tube. The bottle should be closed with a cotton plug to keep out foreign substances. The scale is made by closing this portion of the system and applying suction until the column of mercury rises in the tube as high as it will; then a meter stick is held parallel with the manometer, the zero end being just opposite the surface of the mercury in the bottle, so that a definite point, as 770 mm., can be marked on the background to serve as a guide for the insertion, behind the tube, of a ruled centimeter paper. The scale shows the exact height of the mercury column. In order to determine the pressure in millimeters within the system, the pressure registered by the manometer is subtracted from the barometric pressure. Test out the complete apparatus to make sure that all connections are air tight.

To distil under diminished pressure, fill the Claisen flask *one-third* full of the solution, and evacuate it to a pressure of 30 mm. or less. Then immerse the bulb of the flask at least *two-thirds* of its depth in a water bath and heat to 45–50 ° C. Keep a slow continuous stream of air passing through the liquid by means of the capillary tube. If the solution froths at the beginning, let in a little air through the stop-cock in the side arm of the flask. When distillation is complete, release the pressure by *slowly* opening this stop-cock before turning off the water.

Claisen, L. Beiträge zur Kenntniss der 1, 3- Diketone. *Ann.*, 277, 162–206. Cf. 177–178 (1893). The Claisen flask is illustrated and its use described.
Brewster, J. F. A simple check valve. *Ind. Eng. Chem.*, 15, 32 (1923).
Langley, W. D. Another check valve. *Ind. Eng. Chem.*, 16, 647 (1924).

Expt. 101. *d*-Glutamic Acid Hydrochloride from Wheat Gluten.—*Hydrolysis and precipitation.*—Place 2500–2800 gm. of wheat (*Triticum vulgare*) flour [6] in a suitable container and gradually add tap water, while stirring, until a stiff dough is formed. Knead this thoroughly and allow it to stand under water for at least half an hour. Then wash the starch from the dough by kneading it in a stream of tap water. Tear 1000 gm. of the crude wet gluten into very small pieces; place in a 4-liter round-bottomed Pyrex flask and add 900 cc. of concentrated hydrochloric acid. Hydrolyze by boiling under a reflux condenser on a sand bath for 14 hours, or until the biuret test (Expt. 76) is negative. After completion of the hydrolysis, filter off the acid insoluble humin on a Büchner funnel fitted with two or three thicknesses of filter paper,

[6] Gluten can be washed from a "strong" wheat flour much more readily than from a "weak" flour (Expt. 45, footnote).

and wash with hot distilled water. Concentrate the combined filtrate and wash water under diminished pressure (p. 136) to 650–700 cc., using a 1000 cc. Claisen distilling flask on a boiling water bath. Place the resulting solution in a beaker, cool in an ice mixture, and saturate it at 5–10° C. with *hydrogen chloride*. To prepare a steady stream of this gas, place solid sodium chloride in a 1000 cc. flask; cover it with concentrated hydrochloric acid and allow concentrated sulfuric acid to drop into the mixture from a dropping funnel. Since hydrogen chloride is less soluble in dilute sulfuric acid than it is in water, it escapes. Pass the hydrogen chloride through two 250 cc. Dreschsel gas wash bottles; the first contains concentrated sulfuric acid and the second, which serves as a trap, is so connected that the true inlet tube becomes the exit to the vessel containing the solution to be saturated. To this exit tube attach an absorption tube, which consists of a glass tube having on one end a bulb perforated with a large number of holes. After saturation with hydrogen chloride allow the mixture to stand in a refrigerator for 3 days. The crude glutamic acid hydrochloride separates in crystalline form. To remove the crystals from the mother liquor, filter them on a Büchner funnel, fitted with a linen cloth; drain as dry as possible, finally using the rubber pressure device. Return the crystalline mass to the beaker, mix with sufficient ice-cold concentrated hydrochloric acid to form a thick paste and again filter. Repeat this operation two or three times.

Preparation of filtering cloth.—Use a coarse linen cloth which is cleaned by boiling in dilute sodium hydroxide solution, then thoroughly washed and dried. Plunge the dry cloth into a mixture of 3 parts of concentrated sulfuric acid and 1 part of concentrated nitric acid for 10 minutes. This acid mixture should never be allowed to rise above 40° C. during the treatment of the cloth. Remove the cloth from the mixture and plunge it into a large volume of water so that the heat of dilution will not cause a rise in temperature. Then wash it in running tap water until litmus paper does not give an acid test when pressed against the cloth. This filtering cloth is really gun cotton and must always be *preserved under water*. It can be used to filter concentrated sulfuric acid and hot concentrated hydrochloric acid solutions. The filtering cloth must *never* be thrown into the waste jar but returned to the instructor.

Rubber pressure device.—To remove the last traces of mother liquor or washing solution, place over the top of the Büchner funnel a piece of dental sheet rubber [7] and fasten it securely with a rubber band or hold

[7] "Ideal" rubber dam is put up in rolls 5, 6, and 7 inches wide or in bulk 32 inches wide. It may be obtained from the Atlantic Rubber Manufacturing Corporation, 239–243 4th Ave., New York.

it with the hands. The suction within the flask draws the rubber down against the crystals and sides of the funnel. The operator may also use his hand or a pestle to press against the rubber.

Purification.—Dissolve the impure glutamic acid hydrochloride crystals by heating them in sufficient distilled water to make a concentrated solution; add 20 gm. of the vegetable decolorizing carbon, Norit, and continue to heat the mixture for about 10 minutes; then filter. The filtrate should be clear and amber colored, otherwise repeat the treatment with 10 gm. of the decolorizing carbon. Saturate the solution with hydrogen chloride in the manner previously described to produce precipitation. After saturation allow the mixture to stand in the refrigerator for about 48 hours. One or two such reprecipitations should give a perfectly pure product. Save the filtrate from each reprecipitation in a bottle provided for this purpose. These filtrates can subsequently be reconcentrated and reprecipitated to recover a considerable amount of the glutamic acid which they contain. Filter off the crystals on a Büchner funnel, return them to the beaker, mix with sufficient ice-cold glacial acetic acid to form a thick paste, and again filter. Repeat this operation. Dry the purified crystals in the air for several days, carefully protecting them from dust; then grind to a powder, and finally dry in an oven at 100° C. from 24 to 48 hours, until all traces of acetic acid are removed. What is the yield of *d*-glutamic acid hydrochloride? Ascertain its purity either by Foreman's titration method (Expt. 111), or by making a determination of the amino nitrogen (Expt. 110). For the latter, dissolve 1 gm. of the *d*-glutamic acid hydrochloride in 100 cc. of distilled water.

To prepare free glutamic acid, dissolve 20 gm. of the dry hydrochloride in the least possible volume of distilled water, and add 5.44 cc. of *N* sodium hydroxide solution for each gram. The glutamic acid separates in crystalline form. Allow to stand over night; then filter off the crystals on a Büchner funnel; wash with cold distilled water, until free from chlorides, and dry. If desired, the filtrate may be concentrated to a small volume and a further quantity of glutamic acid obtained. The yield of glutamic acid is 12–14 grams.

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Expt. 102. *l*-Cystine from Human Hair.—Wash human hair [8] thoroughly with hot tap water and dry. Place 3 liters of 20 per cent hydrochloric acid in a 6-liter round-bottomed Pyrex flask and add as large a portion as possible of 1.5 kg. of the dry hair. Hydrolyze the hair by boiling under a reflux condenser, on a sand bath. Add the remainder of the hair as the first dissolves, and continue the hydrolysis for 5 hours, or until the biuret test (Expt. 76) is negative. Then filter off the acid insoluble humin, collecting the filtrate in a precipitating jar; wash the humin with distilled water. Add gradually to the cooled solution a 25–30 per cent solution of commercial sodium hydroxide, stirring occasionally, until the hydrochloric acid is *nearly neutralized*. Either keep the solution cold during the addition of the sodium hydroxide or add it a portion at a time, allowing the solution to cool after each addition. *Care must be taken to avoid making the solution alkaline with sodium hydroxide.* Next add 20 cc. of glacial acetic acid, then, sodium hydroxide solution until the Congo red (Expt. 64) test for mineral acids is entirely negative and the solution is acid to litmus paper. This is best accomplished by using the Congo red solution on a spot plate. Allow the solution to stand at room temperature for 4 or 5 days so that the cystine may separate. Filter on a Büchner funnel and wash with hot distilled water. Dissolve the crude material, which contains some tyrosine, humin, and melanin in addition to the cystine, in 3 per cent hydrochloric acid; and decolorize. For this purpose use the vegetable decolorizing carbon, Norit, or bone black, which has been *completely freed* from calcium phosphate by boiling with 20 per cent hydrochloric acid and then thoroughly washed with distilled water. Use about 10 gm. of Norit for each 100 cc. of solution, heat to boiling for 10 minutes and filter. The filtrate should be water clear and perhaps slightly

[8] This may be obtained from any barber shop.

amber colored, otherwise repeat the treatment. Reprecipitate the cystine from this clear solution by the method previously used, adding sodium hydroxide until the hydrochloric acid is *nearly* neutralized, then follow with glacial acetic acid and sodium hydroxide. Allow the solution to stand 6 hours, filter on a Büchner funnel, wash with hot distilled water, and dry. To further purify the cystine, dissolve it in 5 per cent, by volume, of ammonium hydroxide, and filter. Reprecipitate by adding acetic acid until the solution is neutral to Congo red but acid to litmus paper. Allow to stand 6 hours, filter, wash with hot distilled water to remove the last traces of tyrosine, and dry. Observe the crystals under the microscope. The yield of *l*-cystine is about 4.5 per cent of the weight of hair used. Ascertain its purity by making a determination of either organic sulfur (Expt. 107) or amino nitrogen (Expt. 110). For the latter, dissolve 0.25 gm. of cystine in distilled water containing some hydrochloric acid and dilute to 50 cc. Multiply the result by the factor 0.926 to correct for the abnormal behavior of cystine with nitrous acid.

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*Van Slyke, D. D. A method for quantitative determination of aliphatic amino groups. *J. Biol. Chem.*, 9, 185–207. Cf. 199–200 (1911).

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Merrill, Alice R. T. Experimental studies on cystine. *J. Am. Chem. Soc.*, 43, 2688–2696 (1921).

Schmidt, C. L. A. A method for the preparation of cystin. *Proc. Soc. Exptl. Biol. Med.*, 19, 50–52 (1921).

*Hoffman, W. F., and Gortner, R. A. Sulfur in proteins. I. The effect of acid hydrolysis upon cystine. *J. Am. Chem. Soc.*, 44, 341–360. Cf. 346–347 (1922). The method used here is a slight modification of Folin's method.

Klinke, K. Pigmentstudien im Anschluss an einen Fall von Ringelhaar. *Biochem. Z*, 160, 28–42. Cf. 35 (1925).

Hoffman, W. F. Sulfur in proteins. II. The effect of mild alkaline hydrolysis upon hair. *J. Biol. Chem.*, 65, 251–254 (1925).

Expt. 103. *l*-Tyrosine from Silk Waste.—Place 200 gm. of silk waste [9] in a 3-liter round-bottomed Pyrex flask and add 600 cc. of hydrochloric acid of sp. gr. 1.115. Hydrolyze by boiling gently under a reflux condenser on a sand bath, heated on an electric hot plate, for 12 hours. After completion of the hydrolysis, filter off the acid insoluble humin and wash it with a small quantity of hot distilled water. In order to drive off the hydrochloric acid as completely as possible, concentrate the combined filtrate and wash water under diminished pressure (p. 136) in a 1000 cc. Claisen distilling flask in a boiling water

[9] This material can be supplied by Cheney Brothers, South Manchester, Connecticut.

bath, until a thick paste forms. Take up the residue in 200 cc. of distilled water and again evaporate to a thick paste. Repeat this operation three or four times. After the last concentration take up the residue with 600 cc. of distilled water; add 20 gm. of the vegetable decolorizing carbon, Norit; heat the mixture to boiling for about 10 minutes, and filter. The filtrate should be clear and amber colored, otherwise repeat the treatment with 10 gm. portions of the decolorizing carbon. Neutralize the filtrate with 20 per cent sodium hydroxide solution. A heavy white precipitate, which is tyrosine, separates. Place the mixture in a refrigerator for 2 or 3 days, filter on a Büchner funnel and wash with a small quantity of ice-cold distilled water. Concentrate the filtrate under diminished pressure to at least one-half its volume and cool as before in order to obtain a second crop of crystals. The mother liquor from tyrosine contains glycine and alanine.

To purify the tyrosine, unite the two crops of crystals; place in 3–4 liters of distilled water; boil until dissolved, and then filter. Allow the filtrate to stand over night in a refrigerator, again filter and wash. Evaporate this filtrate to one-half its volume to obtain another crop of crystals. The yield of *l*-tyrosine is 8–10 per cent of the weight of silk used. Ascertain its purity either by Foreman's titration method (Expt. 111) or by making a determination of the amino nitrogen (Expt. 110). For the latter, suspend 0.15 gm. in distilled water, add a small quantity of hydrochloric acid and dip the flask in a hot water bath for a few seconds until the tyrosine is dissolved; then cool and dilute to 50 cc.

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*Abderhalden, E., und Teruuchi, Y. Notiz zur Darstellung von Tyrosin aus Seide. *Z. physiol. Chem.*, 48, 528–529 (1906).

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Warburg, O., und Negelein, E. Über die Oxydation des Cystins und anderer Aminosäuren an Blutkohle. *Biochem Z.*, 113, 257–284. Cf. 278–279 (1921).

Johnson, T. B., and Daschavsky, P. G. Researches on amines. X. The formation of tyramine by decarboxylation of tyrosine produced from silk. *J. Biol. Chem.*, 62, 725–735 (1924–25).

Shelton, E. M., and Johnson, T. B. Researches on proteins. VII. The preparation of the protein "sericin" from silk. *J. Am. Chem. Soc.*, 47, 412–418 (1925).

Expt. 104. *l*-Histidine from Blood Corpuscle Paste.—*Hydrolysis.*—Mix 500 cc. of fresh blood corpuscle paste [10] with 1000 cc. of 37 per cent

[10] The blood corpuscle paste may be obtained from Armour & Company, Chicago, Illinois.

hydrochloric acid in a *weighed* 3000 cc. long-necked, round-bottomed Pyrex flask; hydrolyze by boiling for 30 hours, under a reflux condenser over an electrically heated asbestos bath. Remove the hydrochloric acid by distillation *in vacuo*, at 60° C. from the same flask, and then dry the residue at 100° C. for 2 hours. Weigh the flask and contents and determine the weight of the residue by subtracting the original weight of the flask from that of flask and contents. The weight of the residue is very important because the amounts of mercuric chloride and sodium carbonate used later, depend upon it.

To remove the ammonia, dissolve the residue in 1000 cc. of distilled water, and add calcium oxide until the reddish-brown precipitate assumes a clay color due to the presence of undissolved lime. This procedure insures complete precipitation of the ferric hydroxide and humin. Add 500 cc. of 90 per cent alcohol and distil *in vacuo* at 40° C. until about 800 cc. of distillate have been collected. Filter the residue in the distilling flask on a Büchner funnel to remove humin, ferric hydroxide and excess lime. Wash the precipitate with 2000 cc. of hot saturated solution of calcium hydroxide. The clear amber-colored liquid contains all of the amino acids in the form of calcium salts.

Isolation of tyrosine and leucine.—Dilute the filtrate to 4000 cc., heat on a water bath and add 350 gm. of solid anhydrous sodium carbonate for every 350 grams of residue, remaining after the removal of the hydrocholric acid. Stir until all the sodium carbonate is dissolved, filter on a Büchner funnel and wash with 1 liter of hot distilled water. Transfer the calcium-free filtrate and washings to a 6-liter flask, cool and add 37 per cent hydrochloric acid until neutral to litmus paper. Remove the remainder of the sodium carbonate by the addition of glacial acetic acid until effervescence ceases. Distil *in vacuo* at 50–60° C. until the volume is reduced to about 800 cc. Small quantities of tyrosine and leucine together with a small amount of sodium chloride crystallize out toward the end of the distillation. Place the mixture in the ice-box for 4 days when the tyrosine and part of the leucine will have separated. Filter the crystals on a Büchner funnel and wash with 200 cc. of ice-cold distilled water. The separation of the leucine from the tyrosine and the further purification of these amino acids may be carried out by the method of Habermann and Ehrenfeld.

Precipitation of the mercury compound of l-histidine.—Dilute the filtrate which contains the histidine to 2000 cc. and divide it into 4 equal parts. Transfer each to a 6000 cc. flask and dilute with 1500 cc. of distilled water. Take an amount of solid mercuric chloride, equal to four times the total weight of residue remaining after the removal of the hydrochloric acid, and add one-fourth to each 6000 cc. flask. Heat the

mixture on a water bath until the mercuric chloride has entirely dissolved; a small quantity of a gray-brown flocculent precipitate, which may be removed later, forms. Cool the liquid and add to each portion 3 liters of sodium carbonate solution, which contains 20 gm. of anhydrous sodium carbonate for every 100 gm. of mercuric chloride, previously added. This precipitates the mercury salt of histidine in the form of a flocculent white solid which settles quickly and leaves a clear supernatant liquid. Test a portion of the latter for complete precipitation by adding more sodium carbonate solution. Using a glass siphon, remove the supernatant liquid and wash the precipitate with 4 liters of distilled water, repeating the washing seven times.

Isolation and purification of l-histidine dichloride.—Combine the 4 portions of mercury salt in a 6-liter flask and add concentrated hydrochloric acid until it is dissolved. However, a small quantity of gray-brown precipitate always remains undissolved. Filter the mixture through large folded filter paper. To remove the mercury, saturate the filtrate with hydrogen sulfide gas, under pressure, occasionally testing filtered portions for complete precipitation. Filter on a Büchner funnel, and distil *in vacuo* in a weighed 2-liter distilling flask at 60° C. Remove the water and hydrochloric acid from the resulting pale yellow gum by heating *in vacuo* at 80° C. for 2 hours. Dissolve the dried residue in 60 cc. of concentrated hydrochloric acid by heating on a water bath. Seed with a few crystals of histidine dichloride, stir gently, and place in the ice-box until crystallization is complete. Filter the crystals on a small Büchner funnel and wash, first, with 50 cc. of cold concentrated hydrochloric acid, and later with a cold mixture of 20 cc. of concentrated hydrochloric acid and 20 cc. of ethyl alcohol. Dry at 100° C. The resulting salt is almost pure *l*-histidine dichloride and should weigh 17–19 gm.

If a purer product is desired, dissolve 15 gm. in 10 cc. of hot distilled water and dilute with 100 cc. of 95 per cent ethyl alcohol. Heat on a water bath until the alcohol boils, and filter hot through a small folded filter paper into a small Pyrex Erlenmeyer flask. Place in the ice-box until crystallization is complete; then filter with suction and wash with 50 cc. of cold 95 per cent alcohol. Dry *in vacuo* over sulfuric acid.

Habermann, J., und Ehrenfeld, R. Eine quantitative Methode zur Trennung des Leucins und Tyrosins. *Z. Physiol. chem.*, 37, 18–28 (1902–03).

Knoop, F. Eine Farbenreaktion des Histidins. *Beitr. Chem. Physiol. Path.*, 11, 356 (1908).

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*Hanke, M. T., and Koessler, K. K. Studies on proteinogenous amines. VI.

The preparation of histidine from blood corpuscle paste. *J. Biol. Chem.*, 43, 521–526 (1920).

HUNTER, G. Note on Knoop's test for histidine. *Biochem. J.*, 16, 637–639 (1922).

Expt. 105. *l*-Tryptophane from Casein.—Tryptophane is formed by hydrolyzing casein with trypsin in alkaline solution at pH 8.1. Treat the hydrolyzed material according to the method of Hopkins and Cole, but precipitate it *once* with mercuric sulfate solution. Decompose the precipitate with hydrogen sulfide and filter; then exactly neutralize the sulfuric acid in the filtrate with barium hydroxide, and again filter. Concentrate this filtrate to a small volume in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136). Further purify the solution by extraction with butyl alcohol according to Dakin's method. For this purpose, use the apparatus designed by Fayolle and Lormand for the extraction of a solution with a lighter solvent, and illustrated in Fig. 19. Connect the extraction apparatus at the bottom with a flask containing the butyl alcohol, and at the top, with a reflux condenser. Fill cylinder, *C*, three-quarters to five-sixths full with the aqueous solution. Heat the flask on a sand bath with high sides so as to reduce condensation, and adjust the flame so that the alcohol boils rather rapidly. As the alcohol condenses, it flows down tube, *B*, emerges into cylinder, *C*, traversing the solution to be extracted, and finally siphons over into tube, *T*, and returns to the flask. The extraction is complete when the residual aqueous solution gives a very faint reaction with Rosenheim's formaldehyde test (Expt. 81). By this method, the tryptophane is collected in the butyl alcohol. Cool and allow crystallization to take place. If necessary, remove a portion of the solvent by evaporation under diminished pressure. Filter the crystals and wash them with ether.

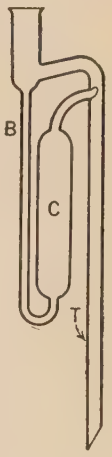

FIG. 19.

*HOPKINS, F. G., and COLE S. W. A contribution to the chemistry of proteids. Part I. A preliminary study of a hitherto undescribed product of tryptic digestion. *J. Physiol.*, 27, 418–428 (1901–02). The isolation of tryptophane from casein is described.

HOPKINS, F. G., and COLE, S. W. A contribution to the chemistry of proteids. Part II. The constitution of tryptophane, and the action of bacteria upon it. *J. Physiol.*, 29, 451–466 (1903).

VAN SLYKE, L. L., and BAKER, J. C. The preparation of pure casein. *J. Biol. Chem.*, 35, 127–136 (1916).

*DAKIN, D. H. On amino acids. *Biochem. J.*, 12, 290–317. Cf. 302–303 (1918).

DAKIN, D. H. Amino acids of gelatin. *J. Biol. Chem.*, 44, 499–529 (1920).

*CLARK, W. M., ZOLLER, H. F., DAHLBERG, A. O., and WEIMAR, A. C. Studies on

technical casein. II. Grain-curd casein. *J. Ind. Eng. Chem.*, 12, 1163–1167 (1920).

*Onslow, H. On the nature of the substances precipitated by mercuric sulfate from hydrolyzed caseinogen, with reference to the estimation and isolation of tryptophan. *Biochem. J.*, 15, 392–399 (1921).

*Fayolle et Lormand, C. Appareil de perforation pour épuisement des liquides par les liquides. Liquides non miscibles. *Chimie & industrie*, 8, 273–274 (1922).

Franzen, H. Extraktionsapparat für grosse Flüssigkeitsmengen. *Z. physiol. Chem.*, 129, 307–308 (1923).

VIII. Synthesis of Amino Acids

Expt. 106. Synthesis of Glycine.—Prepare a solution of 50 gm. of monochloracetic acid in 50 cc. of distilled water and add it slowly with constant shaking, to 600 cc. of ammonium hydroxide solution of sp. gr. 0.90, contained in a flask. Allow to stand for 24 hours. Then concentrate the solution under diminished pressure (p. 136) to one-half of its original volume, but place some sulfuric acid (1 : 2) in both the receiving and guard flasks to react with the ammonia as in the determination of ammonia nitrogen (Expt. 113*d*); this process removes the excess of ammonia. The resulting solution contains glycine and ammonium chloride. To the hot solution add freshly prepared copper carbonate until effervescence ceases, but a slight excess of carbonate remains. A deep blue solution results. Filter and concentrate on the water bath until crystallization begins; then allow to stand for several hours. Copper glycine separates as blue monoclinic crystals; filter on a Büchner funnel; wash with 60 per cent and then with 95 per cent ethyl alcohol, sucking as dry as possible, and finally using the rubber pressure device (Expt. 101). Again concentrate the filtrate on the water bath until crystallization begins.

To prepare the free glycine, dissolve the copper salt in hot distilled water; then decompose it by passing a stream of pure hydrogen sulfide through the hot solution, taking care not to add enough to peptize the precipitated copper sulfide; filter, wash with distilled water containing a little hydrogen sulfide, and suck as dry as possible. Concentrate the filtrate, which contains the free glycine, to a small volume on the water bath and allow crystallization to take place. Filter off the crystals, dry them in the air, pulverize, and again dry at 100–103° C. What is the yield of glycine? Because of the formation of the secondary and tertiary amino acetic acids, $NH(CH_2 \cdot COOH)_2$ and $N(CH_2 \cdot COOH)_3$, the yield is much less than the theoretical. Determine the melting-point (Expt. 128). It should be 228–230° C.

Copper carbonate.—Dissolve 70 gm. of copper sulfate in 150 cc. of hot distilled water and add a saturated sodium carbonate solution, until precipitation is complete. Wash the precipitate several times by decantation, filter on a Büchner funnel, and wash until free from sulfates. Each student should prepare this.

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*Cahours, A. Recherches sur les acides amidés. *Compt. rend.*, 46, 1044–1047 (1858); reprinted in *Ann. Chem. Pharm.*, 107, 147–151 (1858). The author describes the synthesis of glycine from monochloracetic acid and ammonia.

*Kraut, K. Glycocoll und Derivate; von Versuchen von *B. Gollderg, Ph. Kunz* und nach eigenen Versuchen. *Ann.*, 266, 292–310. Cf. 295–299 (1891).

Weyl, T. Die Methoden der Organischen Chemie. Ein Handbuch für die Arbeiten in Laboratorium. Bd. 2, Abteilung 2, S. 890–918. Georg Thieme, Leipzig, 1911.

Ling, A. R., and Nanji, D. R. The synthesis of glycine from formaldehyde. *Biochem. J.*, 16, 702–703 (1922).

Brautlecht, C. A., and Eberman, N. F. Characteristics of the two crystalline forms of glycine. *J. Am. Chem. Soc.*, 45, 1934–1941 (1923).

IX. Estimation of Specific Amino Acids

Expt. 107. *l*-Cystine by Determination of Organic Sulfur.—The determination of sulfur in organic compounds is based upon the oxidation of sulfur to sulfate and the simultaneous destruction of the remainder of the molecule. The oxidation may be accomplished by the use of the Benedict-Denis reagent. The sulfate is subsequently precipitated as barium sulfate. Dissolve 0.15 gm. of *l*-cystine (Expt. 102) in distilled water, containing hydrochloric acid, and dilute to 100 cc. Make duplicate determinations on the solution. Place 10 cc. in an 11.5 cm. porcelain evaporating dish and add from a pipet 10 cc. of the Benedict-Denis reagent. Evaporate the mixture on a water bath to approximate dryness. *Carefully* ignite the contents of the dish with a small flame, gradually increasing the heat to produce a dull redness for a few minutes; this destroys the last trace of organic matter and converts all of the copper nitrate into copper oxide. To avoid spattering during the ignition, remove the evaporating dish from the water bath before its contents are completely dry. Cool, add dilute hydrochloric acid, and warm gently until a clear solution is obtained. Transfer this to a beaker, dilute to 150 cc. with distilled water, heat to boiling and add

sufficient barium chloride solution to precipitate the sulfate as barium sulfate. Allow to stand over night, filter on quantitative filter paper, wash with hot distilled water, and ignite to constant weight in a previously weighed platinum or porcelain crucible. Also make duplicate *blank* determinations on the reagent because it always contains traces of sulfur or sulfates. Subtract the average of the blanks from the average of the original determinations. When using high-grade chemicals, the blank for 10 cc. of the reagent should not exceed 3.5 mg. of barium sulfate. The difference between duplicate determinations of the solution should not be more than 2 mg. of barium sulfate.

If this method is used for the determination of sulfur in other substances than cystine, the solution should be filtered before the addition of barium chloride.

Benedict-Denis reagent.—Dissolve 25 gm. of crystalline copper nitrate, 25 gm. of sodium chloride, and 10 gm. of ammonium nitrate in sufficient distilled water to make a total volume of 100 cc.

*Benedict, S. R. The estimation of total sulfur in urine. *J. Biol. Chem.*, 6, 362–371 (1909).

*Denis, W. The determination of total sulfur in urine. *J. Biol. Chem.*, 8, 401–403 (1910).

Van Slyke, D. D. The analysis of proteins by determination of the chemical groups charactertistic of the different amino-acids. *J. Biol. Chem.*, 11, 15–55. Cf. 28–29 (1911).

Halverson, J. O. The modified Benedict method for the estimation of sulfur in feeds, feces and foods. *J. Am. Chem. Soc.*, 41, 1494–1503 (1919).

Hoffman, W. F., and Gortner, R. A. The determination of sulfur in organic compounds. *J. Am. Chem. Soc.*, 45, 1033–1036 (1923).

Gortner, R. A., and Hoffman, W. F. Quantitative estimation of chlorides and sulfates in expressed plant tissue fluids. *Botan. Gaz.*, 77, 96–102 (1924).

Expt. 108. *l*-Cystine by Uric Acid Reagent.—Folin and Denis discovered that the complete conversion of cystine to cysteine could be accomplished by the addition of a large excess of sodium sulfite; they also found that the mixture gives an intense blue color with their uric acid reagent. Place either 1 gm. of fibrin or 4 gm. of casein in a 300 cc. long-necked Kjeldahl flask. The protein should have been previously dried for 48 hours in a vacuum desiccator over sulfuric acid. Add to the flask 25 cc. of 20 per cent sulfuric acid and insert the condenser, Fig. 20, in the neck; boil the mixture gently for 12 hours on a sand bath, heated either on an electric hot plate or over a burner. Cool, transfer to a 100 cc. calibrated volumetric flask, dilute to volume, and mix thoroughly. Pipet 5 cc. of the fibrin solution or 10 cc. of the casein solution into another 100 cc. volumetric flask and add 20 cc. of saturated sodium carbonate solution, and then 10 cc. of a freshly prepared 20

per cent sodium sulfite solution; shake the mixture thoroughly and allow to stand while the color standards are being made.

From the standard cystine solution prepare two color standards: place in a 100 cc. flask 1 cc., and in another, 3 cc. of the cystine solution; the first thus contains 1 mg. and the second 3 mg. of cystine. To each flask add 20 cc. of saturated sodium carbonate solution, and 10 cc. of 20 per cent sodium sulfite solution; mix thoroughly and allow to stand 5 minutes. Then add to each, with shaking, 3 cc. of uric acid reagent. Also add 3 cc. of the reagent to the hydrolyzed protein solution in which the amount of cystine is to be determined. Allow the three flasks to stand for 10 minutes; then dilute the contents to the mark with distilled water. Using a colorimeter (Expt. 62), make comparisons between the unknown and the standard nearest it in color. Then calculate the number of milligrams of cystine in the sample hydrolyzed, and from this determine the per cent of cystine in the protein.

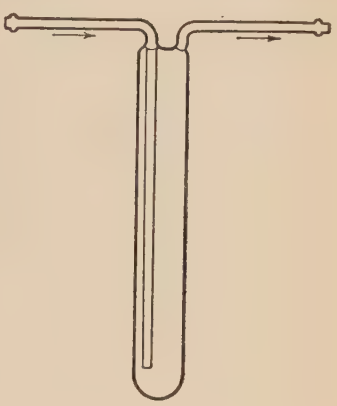

FIG. 20.

Standard cystine solution.—Place 50 mg. of *l*-cystine (Expt. 102) in a 50 cc. volumetric flask and dilute to volume with 5 per cent sulfuric acid. This solution keeps indefinitely.

Uric acid reagent.—Add 50 gm. of sodium tungstate and 40 cc. of 85 per cent orthophosphoric acid to 375 cc. of distilled water. Boil the mixture gently for 2 hours or more in a flask fitted with a reflux condenser, cool, and dilute to 500 cc. with distilled water.

* FOLIN, O., and DENIS, W. On phosphotungstic-phosphomolybdic compounds as color reagents. *J. Biol. Chem.*, 12, 239–243 (1912).

*FOLIN, O., and LOONEY, J. M. Colorimetric methods for the separate determination of tyrosine, tryptophane, and cystine in proteins. *J. Biol. Chem.*, 51, 421–434 (1922).

JONES, D. B., GERSDORFF, C. E. F., and MOELLER, O. The tryptophane and cystine content of various proteins. *J. Biol. Chem.*, 62, 183–195 (1924).

Sullivan has recently described a new color reaction which is claimed to be distinctive for cystine. He has also applied this test to the quantitative determination of cystine. The author has not as yet had the opportunity to test this method.

SULLIVAN, M. X. A distinctive test for cystine. *U. S. Public Health Reports* 41, 1030–1056, May 28, 1926.

Expt. 109. *l*-Tyrosine by Phenol Reagent.—To determine the amount of tyrosine in a protein hydrolysate containing tryptophane, it is first necessary to remove the tryptophane by precipitation with Hopkins and Cole's mercuric sulfate reagent. Sodium cyanide is then used to remove the excess of mercury ions. Place 1 gm. of either casein, fibrin, or Witte's peptone in a 300 cc. long-necked Kjeldahl flask. The protein should have been previously dried for 48 hours in a vacuum desiccator over sulfuric acid. Add to the flask 3.5 gm. of crystallized barium hydroxide and 25 cc. of distilled water; insert a condenser (Expt. 108) in the neck, and boil the mixture gently from 40 to 48 hours on a sand bath, heated either on an electric hot plate or over a burner. Cool, add 30 cc. of 20 per cent sulfuric acid and heat the flask in a boiling water bath from 30 to 60 minutes to drive off any hydrogen sulfide which may have formed. Cool, transfer to a 100 cc. calibrated volumetric flask, dilute to volume, and filter through a *dry* filter paper into a dry flask. The resulting filtrate has about the right degree of acidity for the separation of tyrosine and tryptophane.

Transfer 5–8 cc. of the filtrate to a 15 cc. centrifuge tube, graduated at 10 cc. Add 2 cc. of mercuric sulfate reagent and dilute to 10 cc. with 5 per cent sulfuric acid. Insert a rubber stopper and shake the mixture vigorously and thoroughly. Allow it to stand for 2 hours, remove the stopper and centrifuge. Decant the clear supernatant liquid, which contains the tyrosine, into a *dry* test tube and use for the tyrosine determination. The mercuric tryptophane sediment in the centrifuge tube may be used for the determination of tryptophane.

Pipet into a 100 cc. calibrated volumetric flask, 5 cc. of the supernatant liquid. To prepare the color standard, place in another 100 cc. flask 1 cc. of the standard tyrosine solution, and add 1 cc. of the mercuric sulfate reagent and 3 cc. of 5 per cent sulfuric acid. Then add to each flask 30 cc. of distilled water and 20 cc. of saturated sodium carbonate solution and shake thoroughly; next add 4 cc. of 5 per cent sodium cyanide solution. The yellow mercuric carbonate precipitated by the sodium carbonate dissolves upon the addition of the cyanide. Add to each flask 2 cc. of the phenol reagent, shake thoroughly, and allow to stand from 25 to 30 minutes; then dilute the contents to the mark with distilled water. Using a colorimeter (Expt. 62), make a comparison between the unknown and the standard, which should be set at 40 mm. Then calculate the number of milligrams of tyrosine in the hydrolyzed protein and from this determine the per cent of tyrosine in the sample. At the conclusion of the experiment pour all solutions directly into the drain pipe of the sink on account of the cyanide.

Mercuric sulfate reagent.—Prepare a solution, containing 10 per cent

mercuric sulfate dissolved in 5 per cent sulfuric acid. To do this, thoroughly triturate 10 gm. of mercuric sulfate in a mixture of 50 cc. of distilled water and 5 cc. of concentrated sulfuric acid. Add distilled water to make 100 cc. and filter if necessary.

Standard tyrosine solution.—Place 50 mg. of *l*-tyrosine (Expt. 103) in a 50 cc. volumetric flask and dilute to volume with 5 per cent sulfuric acid.

Phenol reagent.—Boil 15 gm. of molybdic oxide and 10 gm. of sodium hydroxide in 200 cc. of distilled water until the solution no longer smells of ammonia. Then add 100 gm. of sodium tungstate, 50 cc. of 85 per cent orthophosphoric acid, 100 cc. of concentrated hydrochloric acid, and sufficient distilled water to make a total volume of 800 cc. Boil the mixture for 10 hours in a 1.5-liter flask, fitted with a reflux condenser; then add a few drops of bromine to decolorize the solution. Boil off the excess of bromine, cool, filter, and dilute the solution to 1000 cc. with distilled water.

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X. Determination of Aliphatic Amino Groups

Expt. 110. Van Slyke's Method for the Determination of Aliphatic Amino Nitrogen.—This method is based on the measurement of the nitrogen gas liberated in the reaction,

$$RNH_2 + HNO_2 \rightarrow ROH + N_2 + H_2O$$

During the process, the nitrous acid solution decomposes spontaneously resulting in the formation of nitric oxide,

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$

This is used to displace the air in the apparatus; the amino solution is then introduced and nitrogen mixed with nitric oxide is evolved. The oxide is absorbed by alkaline permanganate solution in a modified Hempel pipet, and the pure nitrogen is measured in a special gas buret.

Figure 21 illustrates the manner in which the entire apparatus is arranged, and Fig. 22 shows the details of the deaminizing bulb and the connections.

Description.—"*D* is of 40–45 cc. capacity, *A* of about 35 cc. and the buret *B* of 10 cc. The wire from which the deaminizing bulb *D* is suspended should be fairly stiff, and rigidly fastened in position from above so that the loop about the capillary acts as a fixed center. *A* is then so placed that its center of gravity comes near this center and the shaking of *D* is accomplished with a minimum motion in *A* and, consequently, without putting a dangerous strain on the tube which connects *A* with *D*. This tube is strong-walled and of 3 mm. inner diameter. It is essential that the bore of cock *a* should also be 3 mm. The reason for this is that during the analysis gas containing some nitrogen collects in the tube. Unless *a* is of as wide bore as the tube the liquid from *A* may flow around the bubble instead of forcing it into *D* at the end of the reaction. The cock *d* is also of large bore in order to facilitate emptying *D*. The neck connecting *D* and *B* must be of at least 8 mm. inner diameter in order to allow free circulation of the solution into *D*. The small bulb at the top of *D* keeps the reacting solution from splashing into the capillary."

The connections, joining the deaminizing bulb *D* to the gas buret *F* and this in turn to the Hempel pipet, should be of soft heavy-walled rubber tubing. To insure tightness of the stop-cocks, they should be lubricated with stop-cock grease. Rubber tubing may be used to carry off the overflow and spent solutions, and to protect the hands from nitrous acid. The apparatus may be cleaned by filling the burets and deaminizing bulb with chromic acid cleaning solution. The Hempel pipet should be filled with alkaline potassium permanganate solution, and the gas buret and its connecting reservoir should contain 1 per cent sulfuric acid solution. When the apparatus is not in use, it is well to fill the capillary tube leading from the buret to the Hempel pipet with 1 per cent sulfuric acid from the buret, for manganese dioxide will be deposited if potassium permanganate is left in the tube.

Procedure.—The determination is carried out in three stages:

"1. *Displacement of Air by Nitric Oxide.*—Water from *F* fills the capillary leading to the Hempel pipet and also the other capillary as far as *c*. Into *A* one pours a volume of glacial acetic acid sufficient to fill one-fifth of *D*. For convenience, *A* is etched with a mark to mea-

sure this amount. The acid is run into D, cock c being turned so as to let the air escape from D. Through A one now pours sodium nitrite solution until D is full of solution and enough excess is present to rise a little above the cock into A. It is convenient to mark A for measuring off this amount also. The gas exit from D is now closed at c, and, a being open, D is shaken for a few seconds. The nitric oxide, which

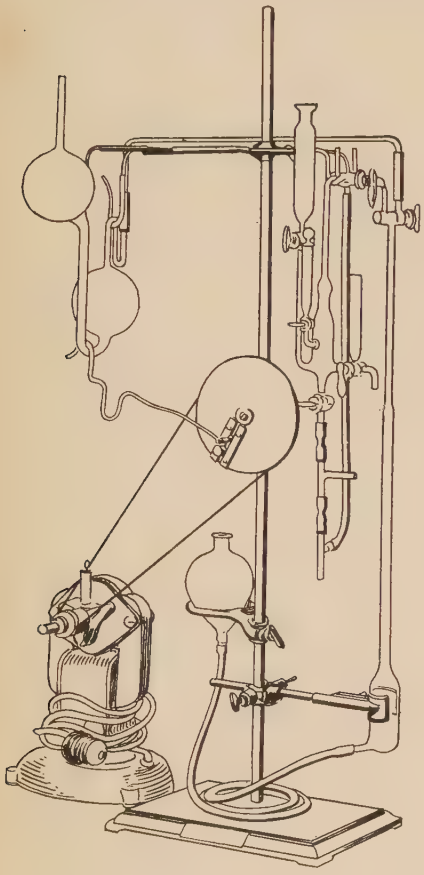

Fig. 21.

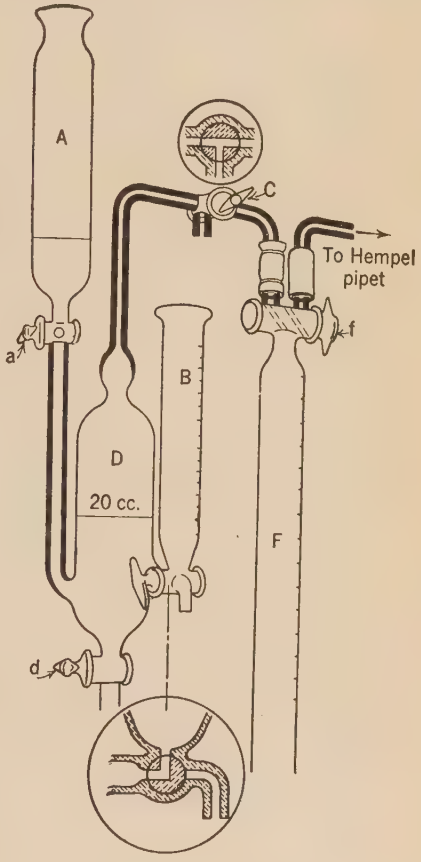

Fig. 22.

instantly collects, is let out at c, and the shaking repeated. The second crop of nitric oxide which washes out the last portions of air, is also let out at c. D is now connected with the motor and shaken till all but 20 cc. of the solution have been displaced by nitric oxide and driven back into A. A mark on D indicates the 20 cc. point. One then closes a and turns c and f so that D and F are connected. The above manipulations require between one and two minutes." During the displace-

ment of air by nitric oxide, the reaction is accelerated by warming the nitrite solution to 30° C. before it is used.

" 2. *Decomposition of the Amino Substance.*—Of the amino solution to be analyzed 10 cc. or less, as the case may be, are measured off in *B*. Any excess added above the mark can be run off through the outflow. tube. The desired amount is then run into *D*, which is already connected with the motor, as shown in Fig. 21. It is shaken, when α-amino acids are being analyzed, for a period of three to five minutes. With α-amino acids, proteins or partially or completely hydrolyzed proteins, we find that at the most five minutes' vigorous shaking completes the reaction. Only in the case of some native proteins which, when deaminized, form unwieldy coagula that mechanically interfere with the thorough agitation of the mixture, a longer time may be required. In case a viscous solution is being analyzed and the liquid threatens to foam over into *F*, *B* is rinsed out and a little caprylic alcohol is added through it. For amino substances such as amino purins, requiring a longer time than five minutes to react, one merely mixes the reacting solutions and lets them stand the required length of time, then shakes about two minutes to drive the nitrogen completely out of solution.

" When it is known that the solution to be analyzed is likely to foam violently, it is advisable to add caprylic alcohol through *B* before the amino solution. *B* is then rinsed with alcohol and dried with ether or a roll of filter paper before it receives the amino solution.

" 3. *Absorption of Nitric Oxide and Measurement of Nitrogen.*—The reaction being completed, all the gas in *D* is displaced into *F* by liquid from *A* and the mixture of nitrogen and nitric oxide is driven from *F* into the absorption pipet. The driving rod is then connected with the pipet by lifting the hook from the shoulder of *d* and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the pipet. The latter is then shaken by the motor for a minute, which, with any but almost completely exhausted permanganate solutions, completes the absorption of nitric oxide. The pure nitrogen is then measured in *F*. During the above operations *a* is left open, to permit displacement of liquid from *D* as nitric oxide forms in *D*." At the end of the experiment, the nitrous solution is drained from *D*, by opening *d*, and *B* is rinsed and dried with a roll of filter paper or with alcohol and ether.

Blank determinations, which are carried out as above except that 10 cc. of distilled water is substituted for the amino substance, must be run on each lot of nitrite used. On a five-minute blank, 0.3–0.4 cc. of gas is usually obtained. If a much larger correction results, the nitrite should be rejected.

The room temperature and the barometric pressure should be observed. The calculation of the weight of nitrogen gas, which corresponds to the volume obtained, may be made by reference to Table X.

Test the method by determining the purity of an amino acid, such as tyrosine. Suspend 0.15 gm. of tyrosine (Expt. 103) in distilled water, add a small quantity of hydrochloric acid and dip the flask in a hot water bath for a few seconds until the tyrosine is dissolved; then cool and dilute to 50 cc.

Stop-cock grease.—Heat together one part of rubber, one part of paraffin, and two parts of vaseline until dissolved.

Alkaline potassium permanganate solution.—Dissolve 50 gm. of potassium permanganate and 25 gm. of potassium hydroxide in sufficient distilled water to make 1000 cc.

Sodium nitrite solution.—Dissolve 90 gm. of sodium nitrite in sufficient distilled water to make 300 cc.

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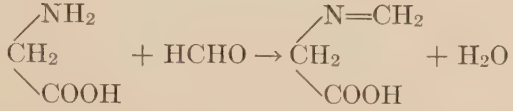
TABLE X

MILLIGRAMS OF AMINO NITROGEN CORRESPONDING TO 1 CC. OF NITROGEN GAS AT 11° TO 30° C.; 728 TO 750 MM. PRESSURE

t	728	730	732	734	736	738	740	742	744	746	748	750	*t*
11°	0.5680	0.5695	0.5710	0.5725	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5840	0.5855	11°
12°	0.5655	0.5670	0.5685	0.5700	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5815	0.5830	12°
13°	0.5630	0.5645	0.5660	0.5675	0.5695	0.5710	0.5725	0.5740	0.5755	0.5770	0.5785	0.5805	13°
14°	0.5605	0.5620	0.5625	0.5650	0.5665	0.5680	0.5700	0.5715	0.5730	0.5745	0.5760	0.5775	14°
15°	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5705	0.5720	0.5735	0.5750	15°
16°	0.5555	0.5570	0.5585	0.5600	0.5615	0.5630	0.5645	0.5660	0.5675	0.5690	0.5710	0.5725	16°
17°	0.5525	0.5540	0.5555	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	17°
18°	0.5500	0.5515	0.5530	0.5545	0.5560	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	18°
19°	0.5745	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5630	0.5645	19°
20°	0.5445	0.5460	0.5475	0.5495	0.5510	0.5525	0.5540	0.5555	0.5570	0.5585	0.5600	0.5615	20°
21°	0.5420	0.5435	0.5450	0.5465	0.5480	0.5495	0.5510	0.5525	0.5540	0.5555	0.5575	0.5590	21°
22°	0.5395	0.5410	0.5435	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	0.5545	0.5560	22°
23°	0.5365	0.5380	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	23°
24°	0.5335	0.5350	0.5365	0.5380	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	24°
25°	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	25°
26°	0.5260	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	26°
27°	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	27°
28°	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	28°
29°	0.5195	0.5210	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	29°
30°	0.5160	0.5175	0.5190	0.5205	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	30°
t	728	730	732	734	736	738	740	742	744	746	748	750	*t*

XI. Determination of Amino Acids by Titration

Expt. 111. Foreman's Titration Method for the Estimation of Amino Acids, Organic Acids and Organic Bases.—Formaldehyde, added to amino acids, reacts with the amino group to form a methylene derivative. If the simplest amino acid, glycine, is used, the reaction is expressed:



The methylene amino acid which results is strongly acid in reaction because the basic properties of the amino group have been destroyed. The importance of this reaction was shown first by Schiff in a series of articles. He thus laid the foundation for a titration method for the quantitative estimation of amino acids, polypeptides, and similar compounds in aqueous solution. The development and perfecting of the method, however, were accomplished by Sörensen and his co-worker, Henriques, but it is now known as Sörensen's formol titration method.

In 1900 Sutton observed that potassium or sodium hydroxide displaces ammonia from ammonium salts in equivalent quantities when in alcoholic solution at room temperature. If sufficient alcohol is present, the liberated ammonia will not form an ionizable compound with the phenolphthalein which is used as an indicator. The acid in an ammonium salt can be titrated, therefore, with standard alkali in alcoholic solution. In addition, Foreman has shown that primary, secondary, and tertiary amines and their allied bases will not form ionizable compounds with phenolphthalein in alcoholic solution if the concentration of the alcohol is more than 80 per cent. Consequently, under this condition, the acid radical of the salts of these bases can be titrated with standard alkali. He has shown also that certain of the amino acids, when in 85 per cent ethyl alcohol, can be titrated quantitatively with standard alcoholic potassium hydroxide solution. The amino acids, dibasic and proline, give low results when titrated in alcohol under these conditions. However, if formaldehyde is subsequently added, the carboxyl groups of these acids will titrate quantitatively. Though Sörensen's method is used by many workers for the titration of amino acids, the method developed by Foreman has certain distinct advantages and is therefore presented. His method has the additional advantage of making possible the titration of organic acids in 85 per cent alcoholic solution.

Preparation of solution.—Dissolve a known weight of the amino acid or of its salt, or of an amino-acid mixture, in carbon dioxide-free distilled water and dilute the resulting solution so that it is approximately 0.1 *N* in relation to the carboxyl groups. In the case of cystine or tyrosine, where the amino acid is very slightly soluble in water, dissolve it in either 0.1 *N* hydrochloric acid, 0.1 *N* sodium hydroxide, or other suitable solvent so that the amino acid will not separate upon the addition of alcohol. When only a small quantity of the amino acid is available, it may be weighed and dissolved in the required amount of solvent and the whole solution used. In order to avoid the absorption of appreciable quantities of carbon dioxide from the air, make all titrations promptly.

Stage 1. *Titration in water.*—Titrate 5 or 10 cc. of the original solution with 0.1 *N* aqueous sodium hydroxide, using phenolphthalein as an indicator. The sodium hydroxide solution used must have been standardized with phenolphthalein. In the case of salts of amino acids, such as histidine dihydrochloride, the titration gives the acid radical; while with dibasic amino acids, such as glutamic acid hydrochloride, both the acid radical and one of the carboxyl groups are obtained.

Stage 2. *Titration in alcohol.*—Transfer 5 or 10 cc. of the original solution to a 250 cc. Erlenmeyer flask, add 10 volumes of 95 per cent ethyl alcohol and three drops of phenolphthalein. Titrate the mixture in daylight over a white plate with 0.1 *N* alcoholic potassium hydroxide solution until a light pink color is produced. The end point is usually quite sharp. Determine the acidity of the alcohol by titrating the same volume as is used in the original titration and make the appropriate correction. The titration value in Stage 2 includes the reading obtained in Stage 1 and represents the exact equivalent of the free organic acids, of the amide, asparagine, and of the amino acids, cystine, tyrosine, histidine, and phenylalanine. These amino acids are not destroyed during the titration and can therefore be recovered.

Stage 3. *Titration in alcoholic formaldehyde.*—Use the solution obtained in Stage 2 for this titration. For each 50 cc. of alcohol used in Stage 2, add 12.5 cc. of neutral formaldehyde solution and titrate to the same end point. Titrate a mixture of neutral formaldehyde solution and alcohol in the same proportions as are used in both stages and apply the correction to the titration in Stage 3. If an amino-acid mixture has been used, the increase in titration value obtained in Stage 3 indicates the amount of dibasic acids and proline present. The total titration value obtained on completing this stage represents the carboyxl groups of all the amino acids, excepting arginine.

First test the method by determining the purity of a specific amino

acid, such a glutamic acid hydrochloride, tyrosine, or histidine dihydrochloride (Expts. 101, 103, and 104), or a mixture of amino acids. For this purpose, dissolve 0.1836 gm. of glutamic acid hydrochloride in 10 cc. of distilled water; or, to 0.1811 gm. of tyrosine add 15 cc. of 0.1 *N* hydrochloric acid and dip the flask in a hot water bath for a few seconds until the tyrosine is dissolved; then cool. To prepare a histidine solution, dissolve 0.1140 gm. of histidine dihydrochloride in 10 cc. of distilled water. Use the whole quantity of solution for each titration and make allowance for any acid or alkali used in dissolving the amino acids.

Pure ethyl alcohol.—Method I.—Alcohol, which is free from aldehydes and practically all peroxides, is produced by the method of Girard and Cuniasse, as described by Rowe and Phelps. To each liter of 95 per cent ethyl alcohol in a glass-stoppered container, add 3 or 4 gm. of pulverized *m*-phenylenediamine hydrochloride and allow the mixture to stand several days. Then distil it slowly; reject the first 25 per cent of the distillate and collect only the next 50 per cent. Alcohol thus prepared gives no color with Schiff's aldehyde reagent in 48 hours. For ordinary purposes, rejection of the first 100 cc. and collection of the next 800 cc. is satisfactory.

Method II.—Alcohol, which is free from both aldehydes and acids, is produced by the method of Dunlap. Dissolve 1.5 gm. of silver nitrate in 3 cc. of distilled water, and add it to each liter of 95 per cent ethyl alcohol in a glass-stoppered container; shake thoroughly and allow to stand until quiescent. Dissolve 3 gm. of potassium hydroxide, purified by alcohol, in 10–15 cc. of warm alcohol; after cooling, pour it slowly into the alcoholic silver nitrate solution, but do not shake. Silver oxide is thrown down as a very fine precipitate, and in this form, the rapid and complete oxidation of the aldehydes is possible. Allow the mixture to stand several days; then either filter, or siphon off the clear supernatant liquid and distil it. Reject the first 100–200 cc. and collect the next 700–800 cc. The distillate is neutral because an excess of alkali has been used.

Schiff's aldehyde reagent.—Dissolve 0.2 gm. of rosaniline hydrochloride in 10 cc. of freshly prepared, cold, *saturated* aqueous solution of sulfur dioxide. Allow the solution to stand several hours until all of the pink color disappears and it becomes colorless or slightly yellow. Then dilute to 200 cc. with distilled water and preserve in a tightly stoppered amber-colored bottle. The reagent is reddened by alkalis, by heating, and by long exposure to the air at room temperature.

0.1 *N Alcoholic potassium hydroxide solution.*—To pure ethyl alcohol, add potassium hydroxide which is free from carbonate. The resulting

solution should be practically colorless; if otherwise, the aldehydes have not been completely removed from the alcohol. Standardize the alcoholic potassium hydroxide solution, using phenolphthalein as an indicator.

Phenolphthalein solution.—Dissolve 0.5 gm. of phenolphthalein in 100 cc. of 50 per cent ethyl alcohol.

Neutral formaldehyde solution.—Dilute one volume of colorless 37–40 per cent formaldehyde with two volumes of distilled water, and neutralize the mixture to phenolphthalein.

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XII. Separation of Protein from Non-protein Nitrogen

Expt. 112. Separation and Estimation of the Amino and Organic Acids in a Plant Sap.—No notes by the author on this experiment have been found.

XIII. Analysis of a Protein

All methods for the analysis of a protein fall into two general groups: one in which part or all of the individual amino acids are actually isolated in crystalline form from the acid hydrolysate, and the other in which the determination of the nitrogen distribution in various fractions of the hydrolysate is made. Among the methods in the first group are those of Fischer, Kossel and Kutcher, and Dakin. In the second group are the Hausmann method, Osborne and Harris' modification of the Hausmann method, and the more recent method of Van Slyke. The Van Slyke method includes the four fractions of the modified Hausmann method as well as a new method for determining aliphatic amino groups by measuring the nitrogen liberated upon treatment with nitrous acid.

The Van Slyke method of analysis for the determination of certain amino acids is limited in its application to pure proteins, to solutions of practically pure proteins, or to protein substances comparatively free from carbohydrates, fibers, fats, etc.

Expt. 113. Analysis of the Protein, Fibrin.—(*a*) *Determination of total nitrogen.* Place 1 gm. of a protein, such as fibrin, in a dry 800 cc. long-necked, round-bottomed Kjeldahl flask and digest with 30 cc. of concentrated sulfuric acid, 10 gm. of potassium sulfate and a crystal of copper sulfate, according to the Kjeldahl method. After the digestion is complete, cool, dissolve the cake of salts in water and dilute to 500 cc. in a calibrated volumetric flask. Distil duplicate 200 cc. portions of the solution and place 60 cc. of an $N/14$ or 0.0714 N solution of acid in the receiving flasks, adding a few drops of 0.4 per cent sodium alizarinsulfonate as indicator. Make all titrations with $N/14$ acid or alkali, because 1 cc. is equivalent to 0.001 gm. of nitrogen. For all subsequent nitrogen determinations use these standard solutions. In every Kjeldahl determination, make a correction for the *blank* on the reagents. Calculate the total nitrogen in one gram of protein and from this the

amount in 3 gm. Make all percentage calculations in subsequent analyses on the basis of the amount of nitrogen in 3 gm. of protein.

Paul, A. E., and Berry, E. H. The Kjeldahl nitrogen method and its modifications. *J. Assoc. Official Agr. Chem.*, 5, 108–132 (1921).

(*b*) *Hydrolysis.*—Place 3 gm. of a protein, such as fibrin, in a dry 300 cc. long-necked, round-bottomed Pyrex Kjeldahl flask, add 75 cc. of hydrochloric acid of sp. gr. 1.115, insert a condenser (Expt. 108) in the neck, and boil the mixture gently for 24 hours, on a sand bath heated either on an electric hot plate or over a burner.

(*c*) *Acid insoluble humin nitrogen.*—After completing the hydrolysis, filter the solution through a quantitative filter paper and wash the residue with *hot* distilled water until free from chlorides. Subject the precipitate and filter paper to a Kjeldahl determination (*a*), using 10 cc. of an $N/14$ solution of a standard acid in the receiving flask. Occasionally some of the humin sticks to the flask in which the hydrolysis is carried out. This must be included in the nitrogen determination; therefore, place in the flask about 5 cc. of concentrated sulfuric acid and heat until the humin has been dissolved. Pour the mixture into the large Kjeldahl flask and rinse the small flask several times with fresh portions of sulfuric acid until the amount of acid necessary for the determination has been added. What is the source of the acid insoluble humin nitrogen?

Gortner, R. A., and Blish, M. J. On the origin of the humin formed by the acid hydrolysis of proteins. *J. Am. Chem. Soc.*, 37, 1630–1636 (1915).

Gortner, R. A. The origin of the humin formed by the acid hydrolysis of proteins. II. Hydrolysis in the presence of carbohydrates and of aldehydes. *J. Biol. Chem.*, 26, 177–204 (1916).

Gortner, R. A., and Holm, G. E. On the origin of humin formed by the acid hydrolysis of proteins. III. Hydrolysis in the presence of aldehydes. II. Hydrolysis in the presence of formaldehyde. *J. Am. Chem. Soc.*, 39, 2477–2501 (1917).

Holm, G. E., and Gortner, R. A. On the origin of humin formed by the acid hydrolysis of proteins. IV. Hydrolysis in the presence of aldehydes. III. Comparative hydrolysis of fibrin and gelatin in the presence of various aldehydes. *J. Am. Chem. Soc.*, 42, 632–640 (1920).

Gortner, R. A., and Holm, G. E. The origin of the humin formed by the acid hydrolysis of proteins. *J. Am. Chem. Soc.*, 42, 821–827 (1920).

Holm, G. E., and Gortner, R. A. The humin formed by the acid hydrolysis of proteins. VI. The effect of acid hydrolysis upon tryptophane. *J. Am. Chem. Soc.*, 42, 2378–2385 (1920).

(*d*) *Ammonia nitrogen.*—Concentrate the filtrate from the acid insoluble humin in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136), in a boiling water bath, until all of the hydrochloric

acid possible is driven off. Dissemble the apparatus and take up the residue in the Claisen flask with 150–200 cc. of distilled water, washing down the sides as the water is added. To get rid of all traces of hydrochloric acid, carefully rinse out the remainder of the apparatus. Place 50 cc. of $N/14$ sulfuric acid in the 1000 cc. receiving flask and 5 cc. in the guard flask, adding to each sodium alizarinsulfonate as indicator. Again set up the apparatus. The outlet tube of the receiving flask must extend below the surface of the liquid in the guard flask. If there is not enough acid to cover the outlet, distilled water may be added. Place 100 cc. of 95 per cent ethyl alcohol in the Claisen flask to prevent foaming at the beginning of the distillation. Also add a 10 per cent suspension of pure calcium hydroxide until there is a slight excess as shown by the turbidity of the mixture and by an alkaline reaction to litmus paper. Close the apparatus and remove the ammonia by distillation under diminished pressure on a water bath at 45–50° C. for half an hour. When the distillation is finished, wash the acid from both receiving and guard flasks into a beaker or Erlenmeyer flask and titrate with $N/14$ alkali. Run a blank on the reagents used and make the proper corrections. Why is it necessary to use a weak base like calcium hydroxide to liberate the ammonia from its salt? What is the source of the *true* ammonia nitrogen in a protein hydrolysate?

Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A. The different forms of nitrogen in proteins. *Am. J. Physiol.*, 23, 180–200 (1908–09).

(*e*) *Acid-soluble humin nitrogen.*—The alkaline mixture remaining in the Claisen flask from the ammonia determination contains all the amino acids as calcium salts. Filter this on a quantitative filter paper and wash the residue with *hot* distilled water until free from chlorides. Subject the precipitate and filter paper to a Kjeldahl determination (*a*), using 10 cc. of an $N/14$ solution of standard acid. What is the source of the acid soluble humin nitrogen?

(*f*) *Precipitation, washing, and dissolving of the basic phosphotungstates.*—Acidify the combined filtrate and washings from the acid-soluble humin by adding a slight excess of hydrochloric acid; concentrate in a 1000 cc. Claisen distilling flask under diminished pressure, in a boiling water bath, to about 100 cc. Wash the solution into a 300 cc. Erlenmeyer flask with distilled water; add 18 cc. of concentrated hydrochloric acid and 15 gm. of phosphotungstic acid dissolved in 50 cc. of warm distilled water. Use a horn or porcelain spatula for handling the phosphotungstic acid. Dilute the mixture to 200 cc. with distilled water; allow the basic phosphotungstate precipitate to settle, and then test the clear supernatant liquid for complete precipitation by the addition

of a few drops of phosphotungstic acid solution. This is a precaution, since the precipitation of the bases must be complete. Heat the contents of the flask in a water bath for at least an hour or until most of the precipitate is dissolved. Invert a small beaker over the flask and place it in a refrigerator for 48 hours. The bases reprecipitate as crystalline or granular phosphotungstates. What amino acids are precipitated by phosphotungstic acid?

Carefully fit a hardened nitrogen-free filter paper, 12.5 cm. in diameter, against the bottom and side walls of a 7.5 cm. Büchner funnel. To do this, fold the outer portion of the filter paper into a number of plaits so that it will fit snugly against the side walls of the funnel Decant the mother liquor onto the filter paper and drain as dry as possible. As a precaution, transfer the filtrate from the filtering flask to a 1000 cc. beaker. Wash the precipitate remaining in the flask 3 or 4 times with a washing solution, containing 2.5 per cent phosphotungstic acid and 3.5 per cent hydrochloric acid, cooled to 0° C. Each time use 12–15 cc., mix thoroughly, allow the precipitate to settle, and decant the supernatant liquid onto the filter, sucking as dry as possible; finally pour the precipitate also upon the filter and rinse out the flask. Any granules of the precipitates which cling to the flask may be allowed to remain as they have already been sufficiently washed, and will be dissolved later. Using a flat-tipped glass rod, stir the precipitate and washing solution on the funnel into a smooth suspension and then drain as dry as possible. Repeat the washing as before, adding the wash solution in a fine stream around the edge of the filter paper, in order to wash it thoroughly. To determine whether the basic phosphotungstates have been completely freed from mother liquor, test successive portions of the washings for calcium as follows: Place 1–2 cc. of a 10 per cent sodium hydroxide solution containing some sodium oxalate, in a test tube; allow a few drops of the washings from the stem of the Büchner funnel to run down the sides of the tube and to form a layer on top of the solution. Shake gently until just enough alkali has mixed with the upper layer to turn it alkaline. If after standing a few minutes there is no trace of turbidity, the washing is complete. Not more than 200 cc. of washing solution should be used for all the washings. Note that the wash solution must be kept as cold as possible since the solubility of the phosphotungstates of the hexone bases is about one-fourth as great at 0° C. as at room temperature. Should the washings come through somewhat turbid, filter through a small filter paper before adding them to the filtrate in the 1000 cc. beaker. Preserve this combined filtrate and washings for the determination of the total nitrogen in the filtrate from the bases (*n*).

Transfer to a 1000 cc. beaker, as completely as possible, the washed basic phosphotungstate precipitate, using a spatula and a stream of distilled water from a wash bottle. Spread the filter paper out on a large watch glass and wash with distilled water made slightly alkaline by the addition of a few drops of 20 per cent sodium hydroxide solution; add these washings to the beaker. This dissolves the portions of the precipitate imbedded in the fibers of the filter paper. Preserve this hardened filter paper for use in the subsequent filtration of barium phosphotungstate. Again using slightly alkaline distilled water, wash or dissolve out any granules of the basic phosphotungstates adhering to the funnel, to the flask in which they were precipitated and to the small filter paper used for the filtration of the turbid washings. Add to the suspension sufficient distilled water to make a total volume of about 600 cc., a few drops of phenolphthalein solution, then 20 per cent sodium hyroxide solution, drop by drop, with constant stirring. As soon as the solution becomes alkaline, discontinue the addition of the alkali until the color fades, and then add more alkali. By repeating this process all of the precipitate is soon dissolved; however, the experimenter should be sure that solution is complete before proceeding. The solution must be slightly alkaline to phenolphthalein at the end, but must not contain more than three or four drops of alkali in excess of the amount required for neutralization. Why should an excess of alkali be avoided?

Dilute the solution to about 800 cc. and precipitate the phosphotungstic acid by the addition of a *slight excess* of a 20 per cent barium chloride solution. Add a few cubic centimeters at a time until a portion tested with sodium sulfate solution shows the presence of an excess of barium, as indicated by the *immediate* formation of a granular precipitate of barium sulfate. Return each portion tested to the beaker. If the solution loses its red color during the precipitation, add two or three drops more of the alkali for the precipitation is not satisfactory unless the solution is slightly alkaline. Why is it necessary to dilute the solution before precipitation of the phosphotungstic acid?

Filter the precipitate of barium phosphotungstate on the same hardened filter paper and Büchner funnel previously used for the basic phosphotungstates. Wash with hot distilled water, according to the procedure given for washing the basic phosphotungstates, until the residue is free from chlorides. *Acidify* the combined filtrate and washings with a slight excess of hydrochloric acid and concentrate the solution to a volume of about 50 cc. in a 1000 cc. Claisen distilling flask under diminished pressure, on a boiling water bath. During concentration, a small quantity of barium phosphotungstate separates. Filter this off on a quantitative filter paper in a small funnel, into a 100 cc. volu-

metric flask and wash with hot water until free from chlorides. Cool the filtrate and washings, and dilute to the mark. Preserve for the determination of the nitrogen of the bases (*h*) (*k*).

(*g*) *Phosphotungstic acid humin nitrogen.*—After washing, subject both the barium phosphotungstate precipitates and filter papers to a Kjeldahl determination. Digest all in the same Kjeldahl flask, continuing this for three hours after the solution becomes clear. When the digestion is carried on for this length of time, the phosphotungstic acid does not interfere with the accuracy of the determination. Use 10 cc. of $N/14$ acid in the receiving flask. The source of the phosphotungstic acid humin nitrogen has not been definitely established.

(*h*) *Total nitrogen of the bases.*—Using 10 cc. portions of the solution containing the bases (*f*), make duplicate nitrogen determinations and from these results calculate the total nitrogen in 100 cc.

To be sure of enough solution for Experiments (*h*)–(*k*), it is advisable to make the determination of arginine nitrogen (*j*), before determining the total nitrogen of the bases; since, if necessary, the same solution may be used for both determinations. Should this be done, to determine the nitrogen of the bases, cautiously add to the solution remaining in the Kjeldahl flask from the arginine determination, 35 cc. of concentrated sulfuric acid, cooling meantime, and a crystal of copper sulfate; digest as in a Kjeldahl determination. Then dissolve the cake of salts in water, transfer the solution to a long-necked Kjeldahl flask, and finish the nitrogen determination. Add the cubic centimeters of $N/14$ acid, neutralized in this determination, to those neutralized in the arginine determination. This sum multiplied by 4 gives the milligrams of total nitrogen in the bases.

(*i*) *Amino nitrogen of the bases.*—Using 10 cc. portions of the solution containing the bases (*f*), make duplicate aliphatic amino nitrogen determinations (Expt. 110), and from the results, calculate the amino nitrogen in 100 cc. Since the ϵ-amino group of lysine reacts slowly towards nitrous acid, the determination must be continued for 30 minutes at 20° C., or longer, if the temperature is lower. The blank determinations must be run for the same length of time. When proteins like the keratins, high in cystine, are used, it is necessary to make a correction for the abnormal behavior of cystine toward nitrous acid.

The difference between the total nitrogen of the bases (*h*) and the amino nitrogen of the bases (*i*) gives the non-amino nitrogen of the bases.

*Van Slyke, D. D. The analysis of proteins by determination of the chemical groups characteristic of the different amino acids. *J. Biol. Chem.*, 10, 15–55 (1911).

Van Slyke, D. D. The quantitative determination of aliphatic amino groups II. *J. Biol. Chem.*, 12, 275–284 (1912).

Van Slyke, D. D., and Birchard, F. J. The nature of the free amino groups in proteins. *J. Biol. Chem.*, 16, 539–547 (1913–14).

(*j*) *Arginine nitrogen.*—The determination of arginine is based on the fact that when boiled with concentrated alkali, it decomposes into one molecule each of ornithine and urea. The urea is then hydrolyzed into carbon dioxide and ammonia, and the amount of the latter determined. The apparatus used is that described by Holm and illustrated in Fig. 23. At the bottom it is connected with an 800 cc. short-necked round-bottomed Kjeldahl Pyrex flask, and the whole apparatus is set up on a Kjeldahl distillation rack. Place in the receiving flask 15 cc. of $N/14$ standard acid, adding sodium alizarinsulfonate as indicator; bring the exit tube as near the surface of the acid as possible. Place in the Kjeldahl flask 12.5 gm. of solid potassium hydroxide, a few pieces of porous porcelain or pumice stone to prevent bumping, and 25 cc. of the solution containing the bases (*f*). Boil the mixture gently for 6 hours, at the same time passing a stream of water through the condenser. At the end of this time, drain the condenser and allow the apparatus to cool. During digestion, the greater part of the ammonia formed passes over into the receiving flask, but a small amount still remains in the Kjeldahl flask. To obtain this, add to the flask, through the separatory funnel, 200 cc. of water, together with a small quantity of zinc dust; and distil off the remaining ammonia as in a regular Kjeldahl determination. Collect the distillate in the same receiving flask as before. Titrate the excess of acid in this flask with $N/14$ alkali. Each cubic centimeter of $N/14$ acid neutralized by ammonia indicates 2 mg. of arginine nitrogen in the aliquot which was taken. From the result, calculate the arginine nitrogen in the basic fraction.

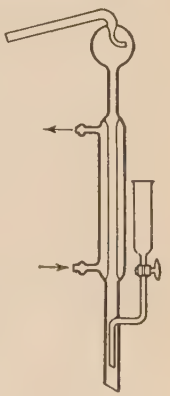

Fig. 23.

Osborne, T. B., Leavenworth, C. S., Brautlecht, C. A. The different forms of nitrogen in proteins. *Am. J. Physiol.*, 23, 180–200 (1908–09).

*Holm, G. E. A modification of the apparatus for the determination of arginine nitrogen by Van Slyke's method. *J. Am. Chem. Soc.*, 42, 611–612 (1920).

(*k*) *Cystine nitrogen.*—Using 10 cc. portions of the solution containing the bases (*f*), make duplicate organic sulfur determinations (Expt. 107), using 5 cc. of the Benedict-Denis reagent. To dissolve the ignited residue, use 10 cc. of 10 per cent hydrochloric acid. Make sure that an excess of barium chloride is present by adding to the diluted boiling solution 10 cc. of 5 per cent barium chloride solution; note that there is some barium chloride already present in the solution containing the bases. Subtract the average of the blanks from the average of these

determinations. Each milligram of barium sulfate indicates 0.06 mg. of cystine nitrogen in the solution analyzed. From this result, calculate the cystine nitrogen in 100 cc. During hydrolysis of the protein, some of the cystine is decomposed, but, according to Gortner and Sandstrom, approximately 65 per cent of the cystine originally present is recovered.

Gortner, R. A., and Sandstrom, W. M. Proline and tryptophan as factors influencing the accuracy of Van Slyke's method for the determination of nitrogen distribution in proteins. *J. Am. Chem. Soc.*, 47, 1663–1671 (1925).

(*l*) *Histidine nitrogen.*—This is obtained by calculation from the arginine nitrogen (*j*) and from the non-amino nitrogen of the bases (*i*) Since arginine contains three-fourths of its nitrogen in non-amino form and histidine contains two-thirds of its nitrogen as non-amino nitrogen, to calculate the histidine nitrogen, subtract three-fourths of the arginine nitrogen from the non-amino nitrogen of the bases, and multiply the difference by three-halves.

That is

$$\text{Histidine N} = \tfrac{3}{2}\text{ (non-amino nitrogen of bases} - \text{arginine N)}$$

or

$$1.5\text{ (non-amino nitrogen of bases} - 1.125\text{ arginine N)}$$

Koessler, K. K., and Hanke, M. T. Studies on proteinogenous amines. II. A microchemical colorimetric method for estimating imidazole derivatives. *J. Biol. Chem.*, 39, 497–519 (1919).

Koessler, K. K., and Hanke, M. T. Studies on proteinogenous amines. VII. The quantitative colorimetric estimation of histidine in protein and protein-containing matter. *J. Biol. Chem.*, 43, 527–542 (1920).

(*m*) *Lysine nitrogen.*—Having thus calculated arginine, cystine, and histidine, lysine may be obtained by subtracting their sum from the total nitrogen of the bases (*h*).
That is

$$\text{Lysine N} = \text{Total N} - (\text{arginine N} + \text{cystine N} + \text{histidine N})$$

(*n*) *Total nitrogen in the filtrate from the bases.*—To the combined filtrate and washings from the basic phosphotungstates contained in the 1000 cc. beaker, add gradually with constant stirring, 30 per cent sodium hydroxide solution until the mixture becomes slightly turbid from the precipitation of calcium hydroxide. *Immediately* add a little glacial acetic acid to dissolve the precipitate and to make the solution slightly acid. Care is necessary in adding the alkali to avoid passing the neutral point by more than a few drops, for otherwise the precipitate may not redissolve. Concentrate the solution to about 150 cc. in a 1000 cc. Claisen distilling flask under diminished pressure, on a boiling water bath; at this point salt may begin to crystallize out. Transfer to a 200 cc. calibrated volumetric flask, cool,

and dilute to the mark with distilled water. Using 25 cc. portions of the solution, make duplicate Kjeldahl nitrogen determinations. To each portion, add 35 cc. of concentrated sulfuric acid, 15 gm. of potassium sulfate, and a crystal of copper sulfate. Add the sulfuric acid carefully under a hood, because of the vigorous evolution of hydrogen chloride gas. Continue the digestion for 3 hours after the solutions have become clear; then the phosphotungstic acid will not interfere with the accuracy of the determination. From the results, calculate the total nitrogen in the 200 cc. of filtrate from the bases.

(*o*) *Determination of amino nitrogen in the filtrate from the bases.*— Duplicate determinations are run in the usual manner for six minutes. A blank determination of the reagents must also be run for the same length of time. The difference between the total nitrogen and the amino nitrogen gives the non-amino nitrogen of the filtrate.

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CHAPTER V

CARBOHYDRATES

MONO- AND DI-SACCHARIDES

I. Color Reactions of the Sugars with Phenols

The color reactions of the sugars with phenols are very distinctive. They are obtained by treating the sugars with different phenols in the presence of concentrated sulfuric or hydrochloric acids. The development of color is due to the formation of condensation products between the phenol derivatives and the decomposition products produced from the sugars by the action of the acid. The reactions of sugars and phenols in the presence of acids were pointed out by both Ihl and Molisch at about the same time. The most important phenol derivative is α-naphthol which is the one employed in the general color reaction for carbohydrates. In using this, condensation takes place in the presence of sulfuric acid. When the condensation is brought about with hydrochloric acid, the solution is usually heated. This is the case in the orcinol and phloroglucinol tests for pentoses and the resorcinol test for ketohexoses. The colors formed at first are very bright, but are not permanent. They rapidly darken and the solution becomes turbid with the formation of a dark-colored precipitate.

Expt. 114. α-Naphthol Test. The Molisch Reaction.—Place 2 cc. of the carbohydrate solution in a test tube; add 2 drops of a fresh 10 per cent solution of α-naphthol in 95 per cent ethyl alcohol or in chloroform, and mix thoroughly. Pour 2 or 3 cc. of concentrated sulfuric acid down the side of the inclined tube so that the acid forms a layer beneath the carbohydrate solution without mixing with it. In a few seconds a purple-red ring will appear at the zone of contact. Either shake the tube well, or allow it to stand a few minutes and observe that the color changes to dark purple. Add 5 cc. of distilled water and note the formation of a dull violet precipitate. Then add an excess of concentrated ammonium hydroxide and observe that the precipitate becomes a rusty yellowish-brown. Is this a specific test for carbohydrates alone? The test is extremely delicate so it is essential that the substance tested should be free from traces of filter paper, that the reagent should

be pure, and the sulfuric acid free from traces of nitrous acid. The reagents may be tested by shaking one drop of α-naphthol solution with ten drops of distilled water and 1 cc. of concentrated sulfuric acid. The mixture should produce a golden yellow color.

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*Mulliken, S. P. A method for the identification of pure organic compounds. Vol. I, p. 26. John Wiley & Sons, New York, 1905. A modification of the Molisch test.

II. Effect of Alkalis on Sugars

Expt. 115. Interconversion of Aldoses and Ketoses.—In the presence of very dilute alkalis, the hexoses undergo a series of complex reciprocal transformations. These were discovered by Lobry de Bruyn and Ekenstein. Decomposition or dissociation does not take place; instead the structural rearrangement of the sugar molecule is affected in such a manner as to produce a number of substances which differ from each other in the position of certain chemical groups within the molecule. This change takes place through the formation of an intermediate enol compound and is often called enolization.

Using a pipet, measure the following solutions into test tubes:

1A. 1 cc. of saturated barium hydroxide solution.
1 cc. of 0.2 *M* glucose solution.

2A. 1 cc. of saturated barium hydroxide solution.
1 cc. of 0.2 *M* fructose solution.

1B. 1 cc. of distilled water.
1 cc. of 0.2 *M* glucose solution.

2B. 1 cc. of distilled water.
1 cc. of 0.2 *M* fructose solution.

Cover the solution in each tube with a layer of toluene about one-fourth inch thick and set aside until the next laboratory period. Take 1 cc. from each of the *A* tubes and place in two other tubes. To these add drop by drop 5 per cent hydrochloric acid until the solutions are neutralized or slightly acid. Add to these tubes and to two others, containing 1 cc. each of the *B* solutions, 1 cc. of resorcinol solution (Expt. 126*b*). Immerse all four tubes in boiling water at the same time. At the end of 5 and 10 minute intervals compare the intensity of the colors in the different tubes. Illustrate by means of structural formulas

the transformations that have taken place. Name the compounds present in the equilibrium mixture. Nef states that this apparent equilibrium is gradually displaced because of the formation of degradation products. What compounds are formed from galactose under similar conditions? Can the members of the *d*-glucose series be transformed into members of the *d*-galactose series?

Lobry de Bruyn, C. A. Action des alcalis dilués sur les hydrates de carbone. I. (Expériences provisoires.) *Rec. trav. chim.*, 14, 156–165 (1895).

*Lobry de Bruyn, C. A., et van Ekenstein, W. A. Action des alcalis sur les sucres. II. Transformation réciproque des uns dans les autres des sucres glucose, fructose et mannose. *Rec. trav. chim.*, 14, 203–216 (1895).

Lobry de Bruyn, C. A., et van Ekenstein, W. A. Action des alcalis sur les sucres. III. Transformation des sucres sous l'influence de l'hydroxyde de plomb. *Rec. trav. chim.*, 15, 92–96 (1896).

Lobry de Bruyn, C. A., et van Ekenstein, W. A. Action des alcalis sur les sucres. IV. Remarques générales. *Rec. trav. chim.*, 16, 257–261 (1897).

Lobry de Bruyn, C. A., et van Ekenstein, W. A. Action des alcalis sur les sucres. V. Transformation de la galactose. Les tagatoses, et la galtose. *Rec. trav. chim.*, 16, 262–273 (1897).

Lobry de Bruyn, C. A., et van Ekenstein, W. A. Action des alcalis sur les sucres. VI. La glutose et la pseudo-fructose. *Rec. trav. chim.*, 16, 274–281 (1897).

Lobry de Bruyn, C. A., et van Ekenstein, W. A. Le *d*-sorbose et le *l*-sorbose (ψ-tagatose) et leur configuration. *Rec. trav. chim.*, 19, 1–11 (1900).

Nef, J. U. On the fundamental conceptions underlying the chemistry of the element carbon. *J. Am. Chem. Soc.*, 26, 1549–1577 (1904).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Erste Abhandlung.) Ueber das Verhalten der Zuckerarten gegen die Fehling'sche Lösung sowie gegan andere Oxydationsmittel. *Ann.*, 357, 214–312. Cf. 294–300 (1907).

Nef, J. U. On the non-equivalence of the four valences of the carbon atom. *J. Am. Chem. Soc.*, 30, 645–650 (1908).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Zweite Abhandlung.) Über das Verhalten der Zuckerarten gegen Ätzalkalien. *Ann.*, 376, 1–119 (1910).

Glatfield, J. W. E. On the oxidation of *d*-glucose in alkaline solution by air as well as by hydrogen peroxide. *Am. Chem. J.*, 50, 135–157. Cf. 137–139 (1913).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Dritte Abhandlung.) *Ann.*, 403, 204–383. Cf. 225 (1914).

*Mathews, A. P. Physiological chemistry. 2nd ed., pp. 858–859. William Wood and Company, New York, 1916.

Expt. 116. Effect on the Reducing Power.—Using a pipet, measure into two test tubes the following solutions in the order indicated:

	A	B
0.2 *M* glucose	5 cc.	5 cc.
0.1 *N* sodium hydroxide	4 cc.	0
0.1 *N* sulfuric acid	0	5.5 cc.

Mix thoroughly the contents of each tube, immerse in boiling water for 10 minutes, cool, and then add:

	A	B
0.1 *N* sulfuric acid	5.5 cc.	0
0.1 *N* sodium hydroxide	0	4 cc.

Both tubes now contain the same amounts of carbohydrate, sodium sulfate, and sulfuric acid. The carbohydrate in tube A has been acted upon by alkali, while that in the tube B has not been exposed to alkali. Decolorize the solution in tube A with a small amount of Norit. Then place 6 cc. portions of A and B in separate test tubes. To each add 6–8 drops of Fehling's solution A (Expt. 118) and heat in a boiling water bath for about 5 minutes. Cool and add to each tube sufficient ammonium hydroxide to make the solution distinctly alkaline. What happens when cupric salts are treated with an excess of ammonia? Explain the differences in behavior of solutions A and B. How does alkali affect the reactivity of the monosaccharides?

*Mathews, A. P. Physiological chemistry. 2nd ed., pp. 857–858. William Wood and Company, New York, 1916.

Expt. 117. Spontaneous Oxidation.—The oxidation of sugars in alkaline solution by means of air has been studied by Framm, who observed that fructose had the greatest speed of oxidation. Mathews studied the rate of oxidation of different sugars in the same medium, using different concentrations of alkali. Place 2 gm. of fructose in a 400 cc. Erlenmeyer flask; add 50 cc. of approximately 0.5 *N* sodium hydroxide solution and close the flask tightly with a one-hole rubber stopper, fitted with a right-angle glass delivery tube about 3–4 mm. in diameter. Attach to this, by means of a rubber tube, another glass tube having a right-angle bend. Place its open end under water. Shake the flask vigorously from 10 to 15 minutes to secure a thorough mixing of the air and the solution. Observe any change in pressure in the apparatus during the shaking. The water will rise in the tube if a negative pressure has developed in the flask. What causes this? How would glucose, galactose, maltose, and lactose behave under similar treatment? Does the same order of oxidation occur in acid solutions?

Framm, F. Über die Zersetzung von Monosacchariden durch Alkalien. *Arch. ges. Physiol.* (*Pflüger's*), 64, 575–599 (1896).

*Mathews, A. P. The spontaneous oxidation of sugars. *J. Biol. Chem.*, 6, 3–20 (1909).

Glattfeld J. W. E. On the oxidation of *d*-glucose in alkaline solution by air as well as by hydrogen peroxide. *Am. Chem. J.*, 50, 135–157. Cf. 152–157. (1913).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Dritte Abhandlung.) *Ann.*, 403, 204–383. Cf. 242–306 (1914).

Spoehr, H. A. The oxidation of carbohydrates with air. *J. Am. Chem. Soc.*, 46, 1494–1502 (1924).

III. Reducing Reactions of the Sugars

Most of the sugars have the property of reducing alkaline solutions of various metallic salts, such as those of copper, silver, mercury, bismuth, tellurium, and others. Application of this fact is made in almost every test for the detection of reducing sugars. However, in the case of Barfoed's solution the reduction of the copper salt is carried out in acid solution, but the reduction is slower. The oxidation of the sugars themselves is very complex. The reduction of alkaline copper solutions is the most important. Fehling's solution contains sodium hydroxide as the base, while the modifications, such as Benedict's and Folin-McEllroy's, contain sodium carbonate; the latter forms solutions considerably less alkaline than the original. These reagents usually contain substances, which hold the cupric hydroxide in solution; for this purpose tartrates, citrates, and phosphates are employed respectively in those mentioned above. Theoretically any aliphatic compound, which contains two or more hydroxyl groups, and which is itself incapable of reducing the copper salt at the boiling temperature, may be used as a substitute for the tartrate employed in Fehling's solution. Phosphates also tend to regulate the degree of alkalinity at a lower hydroxyl-ion concentration than is obtained by carbonates alone. The amount of copper reduced by a given amount of sugar will increase with the hydroxyl-ion concentration.

The solutions for these tests should be preserved with toluene to prevent bacterial action: chloroform should not be used since it reduces the different alkaline copper solutions. Run a blank with each of the reagents. To make this, substitute for the amount of sugar solution used in each test an equal volume of distilled water; add the reagent and perform the test in the usual manner. Compare this result and that obtained with the sugars.

Expt. 118. Fehling's Test.—Prepare 80 cc. of Fehling's solution by mixing, immediately before use, 40 cc. of solutions A and B respectively. Place 8 cc. portions in 9 different test tubes and add respectively, 1 cc. of 0.2 *M* solutions of xylose, rhamnose, glucose, fructose, mannose, galactose, maltose, lactose, and sucrose. Mix the solutions thoroughly; immerse all the tubes in boiling water at the same time and heat for 10 minutes. Reduction is indicated by the formation of cuprous oxide.

If the sugar solution is acid in reaction, it must be neutralized or made slightly alkaline before applying the test.

Place 10 cc. of a 0.2 *M* glucose solution in a test tube, add 1.5 cc. of Fehling's solution and allow the mixture to stand several hours at room temperature. Start this experiment at the beginning of the laboratory period and thereafter observe the color changes which take place. The blue solution soon loses its intensity and becomes an opaque blue-green color. This in turn changes to a leaf-green and then to yellow-green. At this time, more or less yellow precipitate may separate. Later not only the yellow precipitate, but also the supernatant liquid becomes yellow-orange, then brilliant orange and finally bright red. What is the colloidal explanation of these changes?

Soxhlet's modification of Fehling's solution.—Immediately before using, mix equal volumes of A and B.

A. *Copper sulfate solution.*—Dissolve 34.639 gm. of copper sulfate, $CuSO_4 \cdot 5H_2O$, in distilled water, dilute to 500 cc., and if not clear, filter through prepared asbestos (Expt. 139).

B. *Alkaline tartrate solution.*—Dissolve 173 gm. of sodium potassium tartrate (Rochelle salts) and 50 gm. of sodium hydroxide in distilled water, dilute to 500 cc., allow to stand for two days, and if not clear, filter through prepared asbestos.

Fehling, H. Die quantitative Bestimmung von Zucker und Stärkmehl mittelst Kupervitriol. *Ann. Chem. Pharm.*, 72, 106–113 (1849).

Fehling, H. Die quantitative Bestimmung von Zucker. *Ann. Chem. Pharm.*, 106, 75–79 (1858). In this and the preceding article, Fehling has worked out his method with great care; however, he does not recognize the necessity for keeping the solution divided into two parts.

*Soxhlet, F. Das Verhalten der Zuckerarten zu alkalischen Kupfer- und Queck-silber-lösungen. *J. prakt. Chem.*, N. S. 21, 227–317. Cf. 296 (1880); or *Z. anal. Chem.*, 20, 425–451 (1881). The exact conditions, under which the determination must be carried out to get quantitative results, are established.

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Erste Abhandlung.) Ueber das Verhalten der Zuckerarten gegen die Fehling'sche Lösung sowie gegen andere Oxydationsmittel. *Ann.*, 357, 214–312 (1907).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Zweite Abhandlung.) Über das Verhalten der Zuckerarten gegen Ätzalkalien. *Ann.*, 376, 1–119 (1910).

Nef, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Dritte Abhandlung.) *Ann.*, 403, 204–383 (1914).

Fischer, M. H., and Hooker, Marion O. On the colloid chemistry of Fehling's test. *Science*, 45, 505–507 (1917).

*Fischer, M. H., and Hooker, Marion O. Note on the colloid chemistry of Fehling's sugar test. *J. Lab. Clin. Med.*, 3, 368–373 (1918). This article is especially valuable for the description and plate.

Expt. 119. Benedict's Test.—Place 5 cc. of the reagent in each of 9 test tubes and add respectively 1 cc. of 0.2 *M* solutions of the series

of sugars used in Experiment 118. Immerse the tubes in a boiling water bath from 3 to 5 minutes. The resulting precipitates will be red, yellow, or green depending upon the amount of sugar present. This reagent is more sensitive than Fehling's solution.

Benedict's reagent.—Dissolve 173 gm. of sodium citrate and 100 gm. of anydrous sodium carbonate in about 600 cc. of distilled water by heating. If necessary, filter the solution through a fluted filter paper. Dissolve 17.3 gm. of copper sulfate in about 150 cc. of distilled water. Pour the carbonate-citrate solution into a large beaker, and add the copper sulfate solution slowly with constant stirring; then dilute to one liter. The reagent is ready for use and does not deteriorate upon long standing.

*Benedict, S. R. A reagent for the detection of reducing sugars. *J. Biol. Chem.*, 5, 485–487 (1908–09).

Expt. 120. Folin and McEllroy's Test.—Place 5 cc. of the reagent in each of 9 test tubes and add respectively 1 cc. of 0.2 *M* solutions of the series of sugars used in Experiment 118. Heat the tubes in a boiling water bath from 3 to 5 minutes. Minute traces of sugar are indicated by varying degrees of turbidity as in the Benedict test (Expt. 119). This test is very sensitive, is as reliable as Benedict's, and responds more promptly.

Folin and McEllroy's reagent.—Dissolve 100 gm. of U. S. P. sodium pyrophosphate, 30 gm. of crystallized disodium phosphate, and 50 gm. of anhydrous sodium carbonate in about one liter of distilled water. Dissolve 13 gm. of copper sulfate in 200 cc. of water and pour this solution, stirring constantly, into the alkaline phosphate mixture. This reagent keeps indefinitely, except that in cold weather some of the phosphate may crystallize.

*Folin, O., and McEllroy, W. S. Copper-phosphate mixtures as sugar reagents. *J. Biol. Chem.*, 33, 513–519 (1918).

Expt. 121. Barfoed's Test.—Place 5 cc. of the reagent in each of 9 test tubes and add respectively 1 cc. of 0.2 *M* solutions of the series of sugars used in Experiment 118. Place the tubes in a boiling water bath at the same time. At the end of 5 and 10 minute intervals, observe and record the results. This test differs from the other copper reduction tests in that the reduction is brought about in acid solution. Why is the reduction of an acid solution of a cupric salt slower than that of an alkaline solution? When Barfoed's solution is used, is it possible to distinguish the monosaccharides from the disaccharides by a comparison of the rates of reduction.

Barfoed's reagent.—Dissolve 13.3 gm. of crystallized neutral cupric acetate in 200 cc. of distilled water; filter if necessary. Add 5 cc. of

38 per cent acetic acid to the filtrate. The reagent contains about 1 per cent free acid.

*Barfoed, C. Ueber die Nachweisung des Traubenzuckers neben Dextrin und verwandten Körpern. *Z. anal. Chem.*, 12, 27–32 (1873).

McGwigan, H. The oxidation of various sugars and the oxidizing power of different tissues. *Am. J. Physiol.*, 19, 175–198 (1907).

Mathews, A. P., and McGwigan, H. A study of the oxidizing power of cupric acetate solutions. *Am. J. Physiol.*, 19, 199–222 (1907).

Hinkel, F. C., and Sherman, H. C. Experiments upon Barfoed's acid cupric acetate solution as a means of distinguishing glucose from maltose, lactose and sucrose. *J. Am. Chem. Soc.*, 29, 1744–1747 (1907).

*Bunzel, H. H. The rate of oxidation of sugars in an acid medium. *Am. J. Physiol.*, 21, 23–36 (1908).

Bunzel, H. H., and Mathews, A. P. The mechanism of the oxidation of glucose by bromine in neutral and acid solutions. *J. Am. Chem. Soc.*, 31, 464–479 (1909).

Bunzel, H. H. The mechanism of the oxidation of glucose by bromine. *J. Biol. Chem.*, 7, 157–169 (1909–10).

Welker, W. H. A disturbing factor in Barfoed's test. *J. Am. Chem. Soc.*, 37, 2227–2230 (1915). It appears that a very small percentage of sodium chloride interferes with the test.

Expt. 122. Picric Acid Test.—Place 1 cc. of an 0.1 per cent glucose or other reducing sugar solution in a test tube, add 2 cc. of saturated picric acid solution, and 1 cc. of 20 per cent anhydrous sodium carbonate solution. Mix thoroughly, stopper loosely, and heat the tube in a boiling water bath from 20 to 30 minutes. Cool, dilute to 10 cc. with distilled water, shake thoroughly and observe the color. The reduction of picric acid by glucose or other reducing sugar results in the formation of picramic acid, $C_6H_2OHNH_2(NO_2)_2$.

Dehn, W. M., and Hartman, F. A. The picrate colorimetric method for the estimation of carbohydrates. *J. Am. Chem. Soc.*, 36, 403–409 (1914).

Benedict, S. R., and Osterberg, E. A method for the determination of sugar in normal urine. *J. Biol. Chem.*, 34, 195–201 (1918).

Benedict, S. R. A modification of the Lewis-Benedict method for the determination of sugar in the blood. *J. Biol. Chem.*, 34, 203–207 (1918).

Falk, K. G., and Noyes, Helen M. Some observations on colorimetric estimations with solutions containing two colored substances. *J. Biol. Chem.*, 42, 109–130 (1920).

*Rose, A. R. The inversion and determination of cane-sugar. *J. Biol. Chem.*, 46, 529–535 (1921).

Myers, V. C., and Croll, H. M. The determination of carbohydrates in vegetable foods. *J. Biol. Chem.*, 46, 537–551 (1921).

Willaman, J. J., and Davison, F. R. Some modifications of the picric acid method for sugars. *J. Agr. Research*, 28, 479–488 (1924).

Thomas, W., and Dutcher, R. A. The colorimetric determination of carbohydrates in plants by the picric acid reduction method. I. The estimation of reducing sugars and sucrose. *J. Am. Chem. Soc.*, 46, 1662–1669 (1924).

IV. Tests for the Presence of Pentoses

The pentoses exist in plants either in the free state or in the form of condensation products, the pentosans. All the methods for the qualitative detection of pentoses or pentosans depend upon the conversion of the pentose sugars into furfuraldehyde by boiling with a mineral acid, preferably hydrochloric. Furfuraldehyde may be subsequently recognized by the formation of condensation products with a number of substances. Under similar treatment with hydrochloric acid methyl pentoses yield methyl furfuraldehyde, which like furfuraldehyde forms condensation products with some of the same substances, such as phloroglucinol and thiobarbituric acid. The hexoses yield ω-hydroxymethylfurfuraldehyde which also, like furfuraldehyde, forms condensation products and with some of the same substances.

Schindelmeiser, J. Ueber Arabinose in Weidengallen. *Sitzb. nat. Ges. Univ. Dorpat.*, 15, 239–240 (1906). By this investigation the author confirmed the presence of free arabinose in nature.

Davis, W. A., and Sawyer, G. C. The estimation of carbohydrates. IV. The presence of free pentoses in plant extracts and the influence of other sugars on their estimation. *J. Agr. Sci.*, 6, 406–412 (1914).

Englis, D. T., and Hale, C. The occurrence of free pentoses in plants. The effect of extraction of the sugars with ammoniacal alcohol. *J. Am. Chem. Soc.*, 47, 446–449 (1925).

Expt. 123. Orcinol Test.—(*a*) Place 10 mg. of xylose, arabinose, and rhamnose respectively, in each of three test tubes; add 3 cc. of the orcinol solution to each, and heat in a boiling water bath for about 15 minutes. Observe the color changes. Xylose and arabinose develop a green color first, then blue, bluish-violet and finally a grayish-blue turbidity. But rhamnose develops a yellow color, which changes through yellowish-pink to rose. Allow these solutions to stand from 3 to 5 minutes, and then cool under the water tap. Filter each precipitate on a small rapid filter, wash with a little distilled water, and then dissolve it in 95 per cent ethyl alcohol. A green solution is obtained with xylose, a greenish-brown with arabinose, and a rose with rhamnose. For purposes of comparison, perform the test also with a hexose, such as glucose or fructose.

Orcinol solution.—Dissolve 1 gm. of orcinol in 100 cc. of a mixture of equal parts of distilled water and concentrated hydrochloric acid.

*Allen, E. W., und Tollens, B. Ueber Holzzucker (Xylose) und Holzgummi (Xylan.) *Ann.*, 260, 289–306. Cf. 304–306 (1890).

(*b*) Bial found that this test is more sensitive when made in the presence of a little ferric chloride. To 2.5 cc. each of 0.025 *M* xylose,

arabinose, and rhamnose solutions, add 5 cc. of Bial's solution and heat in a boiling water bath for 5 minutes, observing the color changes. Xylose and arabinose develop a green color, which changes through bluish-green and bright blue, to blue, then dark blue turbidity. But rhamnose develops an orange color, which changes through orange-brown and reddish-brown to brown turbidity. This method makes it possible to distinguish a pentose solution from a methyl pentose. The sensitiveness of this test is diminished if hexoses are present.

Bial's solution.—Dissolve 0.5 gm. of orcinol in 250 cc. of concentrated hydrochloric acid, and add 13–15 drops of 10 per cent ferric chloride solution.

*Bial, M. Die Diagnose der Pentosurie. *Deut. med. Wochschr.*, 28, 253–254 (1902).

Bial, M. Bemerkungen zu der Arbeit von F. Sachs: "Farbenreaktionen der Pentosen." *Biochem. Z.*, 3, 323–325 (1907).

van der Haar, A. W. Anleitung zum Nachweis, zur Trennung und Bestimmung der reinen und aus Glukosiden usw. erhaltenen Monosaccharide und Aldehydsäuren. S. 36–39. Gebrüder Borntraeger, Berlin, 1920.

Expt. 124. Phloroglucinol Test.—Place in four different test tubes 1 cc. of a 0.2 *M* solution of xylose, arabinose, rhamnose, and fructose, respectively; add 3 cc. of the phloroglucinol reagent to each tube and heat. Observe the color when the solution reaches the boiling point. Boil one minute and note the darkened color and slight turbidity of the solution. Immediately pour the hot solution on a wet filter paper and wash the precipitate with a little cold 50 per cent ethyl alcohol. The color of the moist precipitate is the characteristic result of the test. The solutions containing xylose and arabinose first show a pure red or violet-red, which rapidly intensifies and darkens. The color of the precipitate varies, according to the duration of the boiling, from a very dark purple to black. In the rhamnose and fructose solutions the first coloration is yellow-orange, which quickly passes through dark orange to dingy brown. The precipitate is rusty brown or a dark uncertain shade of yellow-orange or orange, which changes easily to a dull black if the boiling continues too long.

Phloroglucinol reagent.—Mix 150 cc. of distilled water and 150 cc. of concentrated hydrochloric acid and saturate the solution with powdered phloroglucinol.

Wheeler, H. J., und Tollens, B. Untersuchungen über das Holzgummi. *Ann.*, 254, 320–333. Cf. 329–331 (1889).

Allen, E. W., und Tollens, B. Ueber Holzzucker (Xylose) und Holzgummi (Xylan) *Ann.*, 260, 289–306. Cf. 303–306 (1890).

*Mulliken, S. P. A method for the identification of pure organic compounds. Vol. I, pp. 33–34. John Wiley & Sons, New York, 1905.

Expt. 125. Aniline Hydrochloride Test.—This test may be performed by using either a pentose sugar or a pentosan, which is heated with 12 per cent hydrochloric acid. Place 5 cc. of a 0.2 *M* xylose solution in a 100 cc. Erlenmeyer flask, add 5 cc. of distilled water, and 5 cc. of concentrated hydrochloric acid; or place 0.2 gm. of a pentosan, such as U. S. P. gum acacia (gum arabic), in a 100 cc. Erlenmeyer flask and add 15 cc. of 12 per cent hydrochloric acid. Boil either mixture *gently* from 2 to 5 minutes. While still boiling, place in the mouth of the flask a roll of freshly moistened aniline-hydrochloride paper, and observe. Prepare the paper by wetting a strip of filter paper in an aniline hydrochloride solution, removing the excess by pressing gently between two dry filter papers. A bright crimson appears on the aniline-hydrochloride paper, sometimes in streaks and blotches, but often over the entire surface. Under similar treatment some of the hexose sugars, such as fructose, give a bright pink color. Repeat the experiment, using 5 cc. of a 0.2 *M* sucrose solution, and compare the color produced with that from the pentoses.

Aniline hydrochloride solution.—Mix equal volumes of *freshly* distilled aniline and distilled water, and then add, with constant shaking, concentrated hydrochloric acid until the mixture becomes clear; preserve in an amber-colored bottle.

V. Tests for the Presence of Ketohexoses

Expt. 126. (*a*) **Pinoff's Test.**—This is a modification of the Molisch reaction (Expt. 114), a mixture of ethyl alcohol and sulfuric acid being substituted as the condensing agent. Place in a series of 6 test tubes 1 cc. of 0.2 *M* solutions of fructose, glucose, galactose, mannose, and sucrose respectively, and 1 cc. of 0.01 *M* fructose. To each tube, add 10 cc. of the alcohol-sulfuric acid mixture, and 0.2 cc. of the α-naphthol solution; immerse all the tubes in a boiling water bath and boil for 10 minutes; observe the color changes. In the tubes containing a ketose sugar or a disaccharide, which gives a ketose upon hydrolysis, an intense violet color develops in 2 or 3 minutes. The other sugars tested as well as the pentoses and rhamnose, show no coloration after 10 minutes.

Alcohol-sulfuric acid mixture.—Mix 375 cc. of 95 per cent ethyl alcohol and 100 cc. of concentrated sulfuric acid.

α-Naphthol solution.—Dissolve 2.5 gm. of α-naphthol in 50 cc. of 95 per cent ethyl alcohol.

*Pinoff, E. Uber einige Farben- und Spectral-Reactionen der wichtigsten Zuckerarten. *Ber.*, 38, 3308–3318 (1905).

(*b*) *Resorcinol Test.*—Again using 6 test tubes, place in each respectively the same quantity of a sugar solution as was used in Pinoff's test. Then to each tube add 5 cc. of an alcohol-sulfuric acid mixture (*a*), 5 cc. of 95 per cent ethyl alcohol, and 0.2 cc. of the resorcinol solution. Immerse all the tubes in a boiling water bath and boil for 10 minutes; observe the color changes. In the tubes containing a ketose sugar an intense red color develops in 1 or 2 minutes. The other sugars tested as well as the pentoses and rhamnose, show no coloration after 10 minutes.

Resorcinol solution.—Dissolve 2.5 gm. of resorcinol in 50 cc. of 95 per cent ethyl alcohol.

Seliwanoff, T. Notiz über eine Fruchtzucker reaction. *Ber.*, 20, 181–182 (1887).

Rosin, H. Eine Verchärfung der Seliwanoffschen Reaktion. *Z. physiol. Chem.*, 38, 555–556 (1903). The acid solution is made alkaline with sodium carbonate, shaken with amyl alcohol; the red coloring matter is dissolved, resulting in a greenish fluorescence.

Blanksma, J. J. Over de constitutie van het oxymethylfurfurol. *Chem. Weekblad.*, 6, 1047–1053 (1909). Its constitution is proved to be ω-hydroxymethylfurfuraldehyde.

Van Ekenstein, W. A., and Blanksma, J. J. Über das ω-Oxymethylfurfurol als Ursache einiger Farbreaktionen der Hexosen. *Ber.*, 43, 2355–2361 (1910).

Koenigsfeld, H. Untersuchungen über die physikalisch-chemischen Grundlagen der Seliwanoffschen Lävulosereaktion. *Biochem. Z.*, 38, 310–320 (1912).

(*c*) *Diphenylamine Test. The Ihl-Peckmann-Jolles Reaction.*—Place in a series of 11 different test tubes 1 cc. of 0.2 *M* solutions of fructose, glucose, galactose, mannose, xylose, arabinose, rhamnose, sucrose, maltose, lactose, respectively, and 1 cc. of 0.1 *M* fructose. To each tube add 8–10 drops of diphenylamine solution, and 1 cc. of concentrated hydrochloric acid; immerse all the tubes in a boiling water bath and boil for 10 minutes; observe the color changes. In the tubes containing a ketose sugar, or a disaccharide which gives a ketose upon hydrolysis a deep blue color appears in about 2 minutes; in the aldohexoses a faint blue turbidity develops but in a somewhat longer time; in the pentoses, a green turbidity, and in rhamnose, a cherry red color develop. In each case the color may be extracted with amyl alcohol, and a comparison of the color intensities made; however, the color of rhamnose in amyl alcohol fades almost immediately.

Diphenylamine solution.—Dissolve 20 gm. of diphenylamine in 80 cc. of absolute ethyl alcohol.

Ihl, A. Über die Einwirkung von Diphenylamin auf Kohlenhydrate bei Geganwart von Alcohol, Schwefelsäure oder Salzsäure. *Chem. Ztg.*, 9, 451 (1885).

Jolles, A. Über den Nachweis der Levulose im Harn. *Ber. pharm. Ges.*, 19, 484–486 (1909).

Rasmussen, H. T. B. Über das Verhalten einiger Zuckerarten zu Diphenylamin und Salzsäure. *Ber. pharm. Ges.*, 23, 379 (1913).

Radlberger, L. Zur Kenntnis der Diphenylaminreaktion der Lävulose. *Chem. Zentr.*, 493 (1915).

Expt. 127. Thiobarbituric Acid Test.—Using two test tubes, in one place 20 mg. of fructose and in the other, 20 mg. of xylose; to each add 5 cc. of 12 per cent hydrochloric acid. Heat the fructose solution over a free flame until boiling *begins*, but boil the xylose solution gently from 1 to 3 minutes. Cool both in running water and then add to each 2 cc. of a 12 per cent hydrochloric acid solution, saturated with thiobarbituric acid. Observe the colors that develop. An orange-colored precipitate forms in the fructose solution when it stands for a short time. Allow the xylose solution to stand over night and again observe. What is the condensation product formed?

Dox, A. W., and Plaisance, G. P. A comparison of barbituric acid, thiobarbituric acid and malonylguanidine as quantitative precipitants for furfural. *J. Am. Chem. Soc.*, 38, 2156–2164 (1916).

*Plaisance, G. P. Thiobarituric acid as a qualitative reagent for ketohexose. *J. Biol. Chem.*, 29, 207–208 (1917).

VI. Reactions of Phenylhydrazine

Expt. 128. General Osazone Reaction.—Fischer was the first to show that many sugars react with phenylhydrazine in dilute acetic acid solution to form osazones. Later workers have varied the conditions and made numerous applications of the reaction. Mulliken has based a scheme for the identification of pure sugars upon the time of separation of a precipitate. The appearance of a nearly white crystalline precipitate within less than one minute after heating indicates mannose. All the pentoses and hexoses give a yellow or orange-yellow precipitate from hot solution within 30 minutes. In the case of maltose and lactose, no precipitate separates from the hot solution within 30 minutes, but it appears when the solution is cooled. It is evident that a separation of the osazones of the mono- and di-saccharides can be accomplished by taking advantage of their differences in solubility.

Place in a series of dry test tubes 0.1 gm., respectively, of xylose, arabinose, rhamnose, glucose, fructose, mannose, galactose, maltose, and lactose. Also assign each student one of the sugars in the series, that he may duplicate its reaction. By thus duplicating each sugar, a sufficient quantity of its osazone for purification will be obtained. To each tube, either add 0.2 gm. of pure phenylhydrazine hydrochloride (Expt. 12), 0.3 gm. of crystalline sodium acetate, and 2 cc. of distilled water;

or add 2 cc. of a fresh phenylhydrazine reagent. The errors in measurement should not exceed 1 per cent. Mix the materials thoroughly. Close each tube *loosely* with a cork to prevent evaporation; place it in a tall narrow beaker, containing rapidly boiling water to a depth of 2 or 3 inches, and boil for 30 minutes. A few of the tubes may be immersed at the same time, but it is impossible to perform the experiment using all the tubes at once. Shake each tube occasionally without removing it from the beaker. Any precipitate usually separates quite suddenly. Should this occur, note the exact time which has elasped since the immersion of the tube. Duplicate experiments will generally give results that agree within half a minute. Note the color of the precipitate; whether it is white, yellow, or orange-yellow, and whether it is crystalline, flocculent, or tends to rise to the surface in oily drops. Allow the tubes to cool slowly and note the time when a precipitate forms in those tubes which were clear. Make a table of the different sugars according to the time of osazone formation. This time is greatly modified for mixtures of the different sugars.

What is the object of adding sodium acetate? Write the equations involved in the formation of osazones. Which chemical change is a process of oxidation? What sugars form the same osazone? What does this show in regard to the constitution of these sugars?

Collect the osazones from the two cooled solutions of each sugar on the same filter; wash the monosaccharides four times with 10 cc. of cold distilled water and the disaccharides only once; purify by recrystallization from 60 per cent ethyl alcohol, according to the following method of Fischer. Place the crystals in a test tube and add a small quantity of 60 per cent alcohol; attach a water-jacketed or air reflux condenser and heat in a hot water bath until practically all of the substance has dissolved. At once filter the solution through a small filter paper into a test tube; close this with a stopper, and allow crystallization to take place. Then filter off the crystals by suction on a 5 cm. Büchner funnel; dry first at room temperature on a filter paper covered with a watch glass, and then at 100° C. Observe the crystals under the microscope and make a drawing.

To determine the melting-point, place in a thin-walled capillary tube, 8–10 cm. long, sufficient powdered osazone to make a layer 5 mm. deep. Fasten the tube to a thermometer by a narrow rubber band or a piece of platinum wire, or dip the thermometer in sulfuric acid and place it against the tube so that it adheres by capillary attraction. Then immerse both in concentrated sulfuric acid, contained in a beaker, to a depth equal to half the length of the melting-point tube. Heat the bath, raising the temperature 1° C. every 2 or 3 seconds, and observe

the point at which the osazone melts. To be accurate, it is necessary to make a correction for the exposed mercury column of the thermometer. This is done by the use of the Kopp formula:

$$\text{Correction} = N(T - t)\,\alpha$$

in which N = the portion of the mercury column above the level of the bath or not heated by the vapors, read in degrees;

T = the temperature registered by the thermometer;

t = the average temperature of the exposed column of mercury, obtained from a second thermometer hung so that its bulb is midway between the level of the bath and the top of the mercury column of the first thermometer;

α = 0.000154, the coefficient of apparent expansion of mercury in glass.

Certain thermometers are constructed for very accurate work so that the entire scales are exposed to the vapors of the liquids which are being boiled or distilled. Thus the error due to cooling of the mercury column in the case of the ordinary thermometers is overcome, and the above corrections are unnecessary.

The osazones yielded by the sugars studied, together with their melting-points are given in Table XI.

TABLE XI

Sugar	Osazone	Melting-Point
Xylose	Xylosazone	160° C.
Arabinose	Arabinosazone	160° C.
Rhamnose	Rhamnosazone	180° C.
Glucose	Glucosazone [1]	208° C.
Fructose	Fructosazone	208° C.
Mannose	Mannosazone	208° C.
Galactose	Galactosazone	196° C.
Maltose	Maltosazone	206° C.
Lactose	Lactosazone	200° C.

[1] Glucosamine, or aminoglucose, also yields glucosazone.

Phenlhydrazine reagent.—Mix 2.2 gm. of phenylhydrazine hydrochloride and 3.3 gm. of sodium acetate in 22 cc. of distilled water. Heat to about 60° C. in order to dissolve all substances. Cool before using.

*Kopp, H. Untersuchungen über das specifische Gewicht, die Ausdehnung durch die Wärme und den Siedepunkt einiger Flüssigkeiten. *Ann. Chem. Pharm.*, 94, 257–320. Cf. 262 (1855).

*Fischer, E. Verbindungen des Phenylhydrazins mit den Zuckerarten. *Ber.*, 17, 579–584 (1884).

Fischer, E. Verbindungen des Phenylhydrazins mit den Zuckerarten. II. *Ber.*, 20, 821–834 (1887).

Maquenne. Sur l'emploi de la phénylhydrazine à la détermination des sucres. *Compt. rend.*, 112, 799–802 (1891).

Neuberg, C. Ueber die Reinigung der Osazone und zur Bestimmung ihrer optischen Drehungsrichtung. *Ber.*, 32, 3384–3388 (1899).

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Sherman, H. C., and Williams, R. H. The osazone test for glucose and fructose as influenced by dilution and by the presence of other sugars. *J. Am. Chem. Soc.*, 28, 629–632 (1906).

*Fischer, E. Schmelzpunkt des Phenylhydrazins und einiger Osazone. *Ber.*, 41, 73–77 (1908).

Menge, G. A. A study of melting-point determinations with special reference to the melting-point requirements of the United States Pharmacopoeia. *U. S. Pub. Health Service, Hyg. Lab., Bull.*, 70, 1910.

Browne, C. A. A handbook of sugar analysis, pp. 348–355. Table 24, Appendix, pp. 90–100. John Wiley & Sons, New York, 1912.

Levene, P. A., and LaForge, F. B. On the mutarotation of phenylosazones of pentoses and hexoses. *J. Biol. Chem.*, 20, 429–431 (1915).

Garard, I. D., and Sherman, H. C. A study of the glucosazone reaction. *J. Am. Chem. Soc.*, 40, 955–969 (1918).

VII. Acetylation of Sugars

Acetylation of sugars may be accomplished by the use of acetic anhydride in the presence of a catalyst, such as anhydrous sodium acetate or anhydrous zinc chloride. The aldose sugars are acetylated by acetic anhydride and anhydrous sodium acetate with the application of heat. When either α- or β-aldoses are used, an isomeric change takes place first, and this produces an equilibrated mixture of the α- and β-forms; then acetylation occurs. As a result, the β-pentaacetate predominates because the β-aldoses acetylate more readily than the α-forms. When anhydrous zinc chloride is used as a catalytic agent, the results depend upon whether the temperature is kept high or low; for if low, the α- and β-pentaacetates are produced directly without a previous isomeric change. Therefore, when acetylation is carried out with the pure form of either the α- or β-aldose, the corresponding pentaacetate will be produced. On the other hand, if the temperature is high, an equilibrium mixture of α- and β-pentaacetates is obtained regardless of the form of the aldose acetylated; and in this equilibrium the α-pentaacetate predominates. The pentaacetates are important, as the source from which many other sugar derivatives may be obtained.

Hudson, C. S. The acetyl derivatives of the sugars. *J. Ind. Eng. Chem.*, 8, 380–382 (1916).

Expt. 129. ***β-d*-Pentaacetylgalactose.**—Place 1 gm. of powdered galactose in a large *dry* test tube, add 0.5 gm. of freshly fused sodium acetate and 5 cc. of acetic anhydride. First, carefully heat the mixture over a free flame, shaking to get the materials into solution and to start the reaction; then, close the tube with a cork, and heat in a boiling water bath for 30 minutes. Pour the acetylation product into a casserole, and heat on the steam bath under the hood, adding a few cubic centimeters of 95 per cent ethyl alcohol twice, to aid in driving off the acetic acid and the remaining anhydride. Wash the dark colored residue several times with warm distilled water to remove the sodium acetate; dissolve in hot absolute ethyl alcohol, and decolorize the solution with the vegetable decolorizing carbon, Norit. Filter into a test tube, close with a cork, and allow crystallization to take place. Recrystallize from hot absolute alcohol once or twice. Dry the crystals and determine the melting-point (Expt. 128). This should be 142° C. What compounds are formed by saponification of *β-d*-pentaacetylgalactose with alcoholic potassium hydroxide? What does this experiment show in regard to the number of hydroxyl groups present in the sugar? *β-d*-Pentaacetylglucose, which has a melting-point of 132° C., may be prepared from glucose by this method.

Fused sodium acetate.—This is prepared by fusing crystallized sodium acetate. Place 100 gm. of sodium acetate in a porcelain evaporating dish and heat over a flame. It first melts in its water of crystallization; then becomes solid; then melts again as the temperature rises. When completely melted, pour it into a shallow tin lid or other suitable container, allow to cool, powder, and preserve in a tightly stoppered bottle.

Liebermann, C., und Hörmann, O. Ueber die Formeln des Rhamnetins und Xanthorhamnins. *Ber.*, 11, 1618–1622 (1878). The authors used sodium acetate as a catalytic agent to aid the action of acetic anhydride.

*Erwig, E., und Koenigs, W. Notiz über Pentacetyldextrose. *Ber.*, 22, 1464–1467 (1889).

*Erwig, E., und Koenigs, W. Ueber fünffach acetylirte Galactose und Dextrose. *Ber.*, 22, 2207–2213 (1889).

Hudson, C. S., and Dale, J. K. A comparison of the optical rotatory powers of the alpha and beta forms of certain acetylated derivatives of glucose. *J. Am. Chem. Soc.*, 37, 1264–1270 (1915).

Hudson, C. S., and Johnson, J. M. The isomeric octacetates of lactose. *J. Am. Chem. Soc.*, 37, 1270–1276 (1915).

Hudson, C. S., and Johnson, J. M. The isomeric alpha and beta octacetates of maltose and cellose. *J. Am. Chem. Soc.*, 37, 1276–1280 (1915).

Hudson, C. S., and Dale, J. K. The isomeric pentacetates of mannose. *J. Am. Chem. Soc.*, 37, 1280–1282 (1915).

Hudson, C. S., and Parker, H. O. Conversion of galactose pentacetate to an isomeric form. *J. Am. Chem. Soc.*, 37, 1589–1591 (1915).

HUDSON, C. S. The existence of a third crystalline pentacetate of galactose. *J. Am. Chem. Soc.*, 37, 1591–1593 (1915).

HUDSON, C. S., and JOHNSON, J. M. The isomeric tetracetates of xylose, and observations regarding the acetates of melibiose, trehalose and sucrose. *J. Am. Chem. Soc.*, 37, 2748–2753 (1915).

HUDSON, C. S., and JOHNSON, J. M. A fourth crystalline pentacetate of galactose and some related compounds. *J. Am. Chem. Soc.*, 38, 1223–1230 (1916).

*HUDSON, C. S. The acetyl derivatives of the sugars. *J. Ind. Eng. Chem.*, 8, 380–382 (1916).

VIII. IDENTIFICATION OF CERTAIN MONOSACCHARIDES

Expt. 130. Xylonic Acid Test for Xylose. Bertrand's Reaction.—This test rests on the fact that bromine oxidizes xylose to form xylonic acid. The products of the reaction, xylonic and hydrobromic acids, react with cadmium carbonate to form cadmium xylonate and cadmium bromide. On concentration of the solution, characteristic boat-shaped crystals of the double salt, cadmium bromide xylonate,

$$Cd(C_5H_9O_6)_2 \cdot CdBr_2 \cdot 2H_2O,$$

separate. This is the best test for the detection of xylose in the presence of other sugars.

Place 0.2 gm. of xylose in a test tube, add 1 cc. of distilled water, 0.5 gm. of powdered cadmium carbonate, and 0.25 gm. of bromine (7 or 8 drops). It is essential that an excess of cadmium carbonate should always be present, but too much bromine must be avoided. Warm the mixture slightly, close the test tube loosely with a cork, and allow to stand for 24 hours. Evaporate the solution almost to dryness in a small evaporating dish on a water bath to remove the excess of bromine. Take up the residue in 4–5 cc. of distilled water, filter to remove any unchanged cadmium carbonate, and again evaporate the filtrate almost to dryness. Add about 2 cc. of 95 per cent ethyl alcohol, and allow the mixture to stand. Crystallization will take place in from 3 to 4 hours. If the crystals are not boat-shaped, recrystallize them. Write the equation involved in the test. Examine the crystals under the microscope and make drawings.

*BERTRAND, M. G. Recherches sur quelques dérivés du xylose. *Bull. soc. chim.* [3], 5, 554–557 (1891). For a note on this subject see p. 546–547.

BERTRAND, G. Observations sur quelques nouveaux dérivés de la série des pentoses: l'acide lixonique et la lixite. *Bull. soc. chim.* [3], 15, 592–594 (1896).

*WIDTSOE, J. A., und TOLLENS, B. Ueber Arabinose, Xylose und Fucose aus Traganth. *Ber.*, 33, 132–143. Cf. footnote, 136–137 (1900).

NEF, J. U. Dissoziationsvorgänge in der Zuckergruppe. (Dritte Abhandlung.) *Ann.*, 403, 204–383. Cf. 253–254 (1914).

Expt. 131. Mucic Acid Test for Galactose.—Place 10 cc. of a 0.2 *M* galactose solution, or 20 cc. of a 0.2 *M* lactose solution in a porcelain evaporating dish; add 10 cc. of nitric acid of sp. gr. 1.15, and evaporate the mixture to 2–3 cc., on a water bath at 87° C. Cool, add 5 cc. of distilled water, stir vigorously to start crystallization, and set aside for several days to allow the mucic acid to separate. A fine white crystalline precipitate forms. Examine the crystals under the microscope and make drawings. Filter off the mucic acid on a small filter paper, wash with a little distilled water and dry at 100° C. Determine the melting-point (Expt. 128). This should be 212–215° C. Write the equations for the chemical changes. How do mucic and saccharic acid affect polarized light? This test serves to differentiate galactose and lactose from all other reducing sugars.

KENT, W. H., und TOLLENS, B. Untersuchungen über Milchzucker und Galactose. *Ann.*, 227, 221–232 (1885).

*SCHORGER, A. W., and SMITH, D. F. The galactan of *Larix occidentalis*. *J. Ind. Eng. Chem.*, 8, 494–499 (1916).

Expt. 132. Saccharic Acid Test for Glucose.—The oxidation by strong nitric acid of glucose and the substances which yield glucose upon hydrolysis, produces saccharic acid, which may be recognized by means of its potassium hydrogen or silver salt. This is one of the best tests for *d*-glucose in the presence of other sugars. Place 0.5–1.0 gm. of glucose in a porcelain evaporating dish, add 12 times its weight of nitric acid of sp. gr. 1.15, and evaporate the mixture to a thick sirup, or about 1 cc., on a boiling water bath, with constant stirring. Dissolve the sirup in 10 cc. of distilled water and again evaporate to about 1 cc. Again take up the sirup with a little distilled water, heat gently, add powdered potassium carbonate until the solution is just alkaline to litmus paper, and acidify with acetic acid. Allow to stand over night to permit the potassium hydrogen saccharate to separate. If crystallization does not take place after standing for this length of time, concentrate the solution further by gentle evaporation. Filter off the crystals on a small filter and recrystallize twice from the smallest possible volume of hot distilled water. This process of recrystallization should eliminate the last traces of oxalic acid and other impurities. Observe the crystals under the microscope and make drawings. Filter, dry, and weigh the crystals.

Further identify the potassium hydrogen saccharate, by converting it into the silver salt. To do this, dissolve the crystals in a small quantity of distilled water, neutralize the solution with ammonium hydroxide, and add, with stirring, a silver nitrate solution, which contains a quantity of silver nitrate equal to 1.5 times the weight of the potassium hydro-

gen saccharate used. Allow to stand over night, filter the silver saccharate, $C_6H_8O_8Ag_2$, on a previously prepared and weighed Gooch crucible (Expt. 139), wash free from silver nitrate with a small quantity of distilled water, dry in the dark in a vacuum desiccator over sulfuric acid and weigh. After ignition the weight of silver should equal 50.86 per cent of the weight of silver saccharate used.

*Sohst O. und Tollens, B. Über krystallisirte Zuckersäure (Zuckerlactonsäure.) *Ann.*, 245, 1–27 (1888).

*Gans R., und Tollens, B. Ueber die Bildung von Zuckersäure als Reaction auf Dextrose.—Raffinose enthält Dextrose. *Ann.*, 249, 215–227. Cf. 218–219 (1888).

van der Haar, A. W. Anleitung zum Nachweis, zur Trennung und Bestimmung der reinen und aus Glukosiden u.s.w. erhaltenen Monosaccharide und Aldehydsäuren, S. 100–103. Gebrüder Borntraeger, Berlin, 1920.

Expt. 133. Phenylhydrazone Test for Mannose.—Dissolve 0.3 gm. of mannose in 10 cc. of distilled water in a test tube; add 0.5 cc. of glacial acetic acid, 1 cc. of pure phenylhydrazine (Expt. 12, footnote), 3 cc. of distilled water and shake. Allow to stand in an ice mixture at a temperature of about 10° C. for 2 or 3 hours; shake frequently. Filter off the mannose phenylhydrazone on a small filter paper, wash with a little cold distilled water, and dry at 100° C. for an hour. Determine the melting-point (Expt. 128). This should be 188° C. The hydrazone may be used for the quantitative determination of mannose. For this, filter the hydrazone on a weighed Gooch crucible (Expt. 139); wash several times with a small quantity of cold distilled water, and dry at 100° C., cool in a desiccator, and weigh. The weight of mannose phenylhydrazone multiplied by the factor 0.666 gives the weight of mannose. How may the mannose be regenerated from the hydrazone?

*Fischer, E., und Hirschberger, J. Über Mannose. I. *Ber.*, 21, 1805–1809 (1888).

Fischer, E., und Hirschberger, J. Über Mannose. II. *Ber.*, 22, 365–376 (1889).

de Witt, D. Verfahren zur Darstellung von Mannose. *Z. Ver. Rubenzucherind.*, 32, 794–795 (1895). The author regenerates mannose from its phenylhydrazone by boiling with benzaldehyde.

Herzfeld, A. Ueber die specifische Drehung der Acetylmaltose und Maltose. *Ber.*, 28, 440–443. Cf. 442–443 (1895).

*Bourquelot, E., et Hérissey, H. Sur le dosage du mannose mélangé à d'autres sucres. *Compt. rend.*, 129, 339–341 (1899).

Ruff, O., und Ollendorff, G. Verfahren zur Reindarstellung und Trennung von Zuckern. *Ber.*, 32, 3234–3237 (1899). The authors use formaldehyde to separate sugars from the hydrazones of substituted phenylhydrazines.

Browne, C. A. A handbook of sugar analysis. pp. 347–348. John Wiley & Sons, New York, 1912.

Expt. 134. Methylphenylosazone Test for Fructose.—Dissolve 0.2 gm. of fructose in 10 cc. of distilled water in a test tube; add 0.6 gm. of *asym*-methylphenylhydrazine, and sufficient 95 per cent ethyl alcohol to give a clear solution. Since the fructose may not be chemically pure or other sugars may be present in the solution, warm it slightly and allow it to stand 24 hours so that any insoluble hydrazones may separate; then filter them off. Add 4 cc. of 50 per cent acetic acid to the filtrate; it will at once become yellow in color. Heat the solution in a water bath from 5 to 10 minutes, and allow to stand in a cool dark place for 24 hours. If crystallization has not begun at the end of this time, either *seed* with methylphenylosazone or scratch the sides of the test tube with a glass rod and allow to stand a day or two longer. Filter the crystals and wash, first with distilled water, and then with ether. The latter dissolves the greater part of the adhering oil and the strongly colored by-products. Recrystallize the orange-colored methylphenylosazone from hot benzene, and determine the melting-point (Expt. 128). This should be 152° C. The aldohexose sugars do not form osazones with methylphenylhydrazine because it does not act as an oxidizing agent.

*Fischer, E. Ueber die Verbindungen des Phenylhydrazins mit den Zuckerarten. V. *Ber.*, 22, 87–97. Cf. 91–92 (1889).

*Neuberg, C. Ueber die Isolirung von Ketosen. *Ber.*, 35, 959–966. Cf. 960–961 (1902).

Neuberg, C. Ueber die Isolirung von Ketosen. II. *Ber.*, 35, 2626–2633 (1902).

Neuberg, C. Die Methylphenylhydrazinreaction der Fructose. *Ber.*, 37, 4616–4618 (1904).

IX. Identification of a Disaccharide

Expt. 135. Sucrose in the Presence of Reducing Sugars.—Prepare a solution containing 1 cc. of a 0.2 *M* sucrose solution and 1 cc. of a 0.2 *M* glucose or other reducing sugar solution; add 6 cc. of Fehling's solution (Expt. 118) and boil. Filter off the cuprous oxide, add to the filtrate more Fehling's solution and boil again. If no reduction takes place, the reducing sugars have been removed. If, however, reduction occurs, filter off the cuprous oxide and again boil with more Fehling's solution. Continue this process until all the reducing sugars have been removed. Add hydrochloric acid to the filtrate until it is neutralized; then add 1 or 2 drops of concentrated hydrochloric acid and boil for 2 or 3 minutes. Cool, neutralize with sodium hydroxide solution, again add Fehling's solution, and boil. The formation of cuprous oxide indicates that glucose and fructose have been formed from the sucrose by hydrolysis.

X. Polarimetric Analysis of Sugars

Expt. 136. Specific Rotation.—The specific rotation or specific rotatory power of any substance may be defined as the rotation in angular degrees produced by a length of 1 dm. of a solution containing 1 gm. of substance in 1 cc. When dealing with a homogeneous active *liquid*, the specific rotation is represented by the formula:

$$[\alpha] = \frac{a}{ld}, \quad \ldots \ldots \ldots \quad (1)$$

in which α = the specific rotation;
a = the degrees of angular rotation;
l = the length of the column of solution in decimeters;
d = the density of the solution.

When, on the other hand, the active substance is *in solution*, the concentration must be taken into account; the formula then becomes

$$[\alpha] = \frac{100a}{lc}. \quad \ldots \ldots \ldots \quad (2)$$

in which c = grams of the substance in 100 cc. of solution

or

$$[\alpha] = \frac{100a}{lpd}, \quad \ldots \ldots \ldots \quad (3)$$

in which p = number of grams of the substance in 100 gm. of the solution, or per cent concentration of the substance.

That is $pd = c$ of the preceding formula. Equation (1) is used in determining the specific rotation of a liquid, such as an essential or volatile oil; while equations (2) and (3) are used in determining the specific rotation of sugar solutions.

Influence of variable factors on specific rotation.—In order to determine the correct value of the specific rotation, certain variables must be taken into consideration; these are, the wave length of the light used, the temperature, and the concentration of the substance. It is generally expressed in terms, either of the bright yellow D lines of the sodium spectrum, or of the yellow-green mercury line, λ5461A, at a given temperature. That is, specific rotation, observed by either source of light at 20° C., would be expressed by the symbols,

$$\left[\alpha\right]_D^{20} \quad \text{or} \quad \left[\alpha\right]_{\lambda 5461A}^{20}$$

In determining the specific rotation of sugar solutions it will be observed that most sugars show a decrease in specific rotation with an

increase in temperature, the change being especially marked with fructose and arabinose. Xylose, however, shows a slight increase, while glucose remains apparently unchanged between 0° and 100° C. The specific rotation of fructose decreases until, at 87.3° C. it is equal and opposite to that of glucose. Hence, at this temperature invert sugar becomes optically inactive. This principle is used in the determination of certain sugar mixtures.

The variation in the specific rotation of sucrose and *d*-glucose, in which p is the per cent concentration, is given in the following formulas:

$$\text{Sucrose } \left[\alpha\right]_D^{20} = 66.412 + 0.012673p - 0.0003766p^2.$$

$$d\text{-Glucose } \left[\alpha\right]_D^{20} = 52.50 + 0.0188p + 0.000517p^2.$$

The values of the specific rotations of the two sugars at 5 per cent intervals of concentration are given in Table XII.[2] The specific rotation for other concentrations may be calculated from this table by interpolation.

TABLE XII

Percentage	Sucrose	*d*-Glucose
5	66.466	52.607
10	66.501	52.740
15	66.517	52.898
20	66.515	53.083
25	66.493	53.293
30	66.453	53.529
35	66.394	53.791
40	66.316	54.079
45	66.220	54.393
50	66.104	54.732

Determination of angular rotation on polarimeter and saccharimeter.— The angular rotation may be read directly from a *polarimeter* or may be calculated from the readings on a *saccharimeter*, by using the proper conversion factor. The former is an instrument adapted to the examination of *all* optically active substances. It is provided with a circular scale, divided into angular degrees, with a vernier for greater accuracy in reading. The compensation is accomplished by rotation of the analyzer. The saccharimeter is provided with an arbitrary scale which depends for its value upon the linear movement of a quartz wedge by

[2] This table and the formulas for specific rotation of sucrose and *d*-glucose are taken from *Bur. Standards, Circ.*, 44, pp. 149 and 150, 1918.

which the rotation of a sugar solution is compensated. The polarizer and analyzer remain fixed. The scale expressing angular rotation on the polarimeter is replaced on the saccharimeter with one graduated according to the decimal system which gives the percentage of sugar directly.

For example, the Schmidt and Haensch saccharimeter is equipped with a double wedge compensator, consisting of two movable quartz-wedges, each provided with a scale. These are read through the same telescope, as illustrated in Fig. 24. In the illustration, K is the control and A the working scale, which is set at 85.5°. When using the instrument for dextrorotatory substances, scale K is set at zero with its vernier, and the optical rotation is measured upon scale A; for levorotatory substances, A is set at zero and scale K is used for the reading. A zero-point correction must be made where very great accuracy is desired.

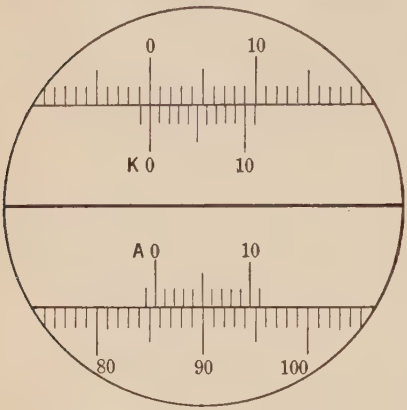

Fig. 24.

Light sources and absorption cell.—The types of lamps used in polarimetry may be divided into two classes, those giving monochromatic or nearly monochromatic light and those giving a white light. To the first class belong the sodium flame and the yellow-green mercury line, λ5461A; to the second belong gas and electric lights. When white light is used, it is necessary to place a dichromate absorption cell between the lamp and the instrument, close to the latter, in order to correct the difference in rotation dispersion between the sugar solution and the quartz-wedge system. The cell eliminates by absorption most of the shorter waves from the visible spectrum. The International Congress for Uniform Methods of Sugar Analysis at the New York meeting in September, 1912, adopted the suggestion of Bryan that white light be passed through a potassium dichromate solution of such strength that the per cent concentration multiplied by the thickness in millimeters is equal to 90. For example, if a 6 per cent potassium dichromate solution is used, the cell should be 15 mm. thick.

The international sugar scale and normal weight.—The International Commission for Uniform Method of Sugar Analysis at the Paris meeting in 1900 recommended a new definition of the 100° point of the saccharimeter, based upon true cubic centimeters and a standard temperature

of 20° C.; as a result the *normal weight* of sucrose became 26.000 gm. The international sugar scale is then defined as follows: All polarizations must be made at 20° C. The graduation of the saccharimeter must also be made at 20° C. 26 gm. of pure dry sucrose, weighed in the air with brass weights, dissolved in distilled water and the volume made up to 100 metric cubic centimeters at 20° C. must give a saccharimeter reading of exactly 100.00.

The international sugar scale is used upon the Schmidt and Haensch, Peters, and Bates type of Fric saccharimeters. To convert the readings on any of these saccharimeters into angular degrees on the polarimeter, 1° S (international sugar scale) = 0°.34620 angular rotation D, according to Bates and Jackson of the Bureau of Standards, or 1° S = 0°.34657, according to Herzfeld and Schönrock.

Determination of the specific rotation of sucrose.—Dissolve 3.25 gm. of sucrose in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C.; use a saccharimeter and make observations in a 200 mm. polariscope tube at 20° C. Take the average of a series of six or eight concordant readings and from this calculate the specific rotation of sucrose. How does this result correspond with the accepted value, +66.5°? When determining the specific rotation of a sugar, mutarotation (Expt. 137) must always be taken into account. Sucrose is one of the few sugars which does not show mutarotation.

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Zerban, F. W. The specific rotation of invert sugar and the Clerget divisor. *J. Am. Chem. Soc.*, 47, 1104–1111 (1925).

Expt. 137. Mutarotation.—When aldehydic, ketonic, or other sugars showing aldehydic functions are freshly dissolved in water, their specific rotation slowly changes, sometimes increasing but generally

decreasing until a constant value is reached. This phenomenon is called mutarotation. It is explained by the fact that each of these sugars exists in two modifications, designated as α and β forms. When they are in equilibrium, constant rotation results. This may be obtained by allowing the sugar solution to stand at room temperature for several hours. Since both alkalis and acids increase the velocity of mutarotation, equilibrium may be brought about in from 10 to 15 minutes by the addition of either 0.1 per cent ammonium hydroxide or 0.005 *N* potassium hydroxide solution; the former method of producing equilibrium is, however, to be preferred. What would be the effect of using stronger alkali? Sugars which show mutarotation also have the power of combining with phenylhydrazine, and of reducing Fehling's solution.

To observe mutarotation, dissolve 2 gm. of *d*-glucose in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube either in a polarimeter or a saccharimeter (Expt. 136). Take the initial reading *immediately* and observe every 20 minutes until three or four readings have been taken; then, either allow the solution to stand over night so that equilibrium may be reached, or add 1 or 2 drops of concentrated ammonium hydroxide solution to the polariscope tube, shake thoroughly, and, in 20 minutes, again take a series of readings. From the results obtained, calculate both the initial and final specific rotations of *d*-glucose, and compare the results with the data given in Table XIII, which includes the rotatory powers of a number of the mutarotating sugars.

TABLE XIII

Sugar	Specific Rotation in Water		
	α Form	Const. Rot.	β Form
d–Xylose	+ 92.0	+ 19.0	− *20.0*[3]
d–Arabinose	− *54.0*	−105.0	−175.0
l–Rhamnose	− 7.7	+ 8.9	+ *54.0*
d–Glucose	+113.4	+ 52.2	+ 19.0
d–Galactose	+144.0	+ 80.5	+ 52.0
d–Mannose	+ 34.0	+ 14.6	− 17.0
d–Fructose	− *21.0*	− 92.0	−133.5
Lactose	+ 90.0	+ 55.3	+ 35.0
Maltose	+*168.0*	+136.0	+118.0
Sucrose[4]	.....	+ 66.5	.....
Invert sugar	.....	− 20.0	.....
Raffinose	.....	+105.2	.....

[3] All calculated values are in italics.

[4] The specific rotations of sucrose, invert sugar, and raffinose are included for purposes of comparison.

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Lowry, T. M. Studies of dynamic isomerism. Part XVIII. The mechanism of mutarotation. *J. Chem. Soc.*, 127, 1371–1385 (1925).

XI. Double Polarization Methods

Expt. 138. Determination of Sucrose.—*Direct polarization.*—Weigh into a small tared dish 13 gm. of a commercial sirup. Transfer the sirup to a 100 cc. volumetric flask using 50 cc. of distilled water; decolorize carefully by adding *basic* lead acetate solution, avoiding an excess. Dilute to 100 cc., mix thoroughly, and filter quickly through a dry filter paper, rejecting the first 15 cc. Cover the funnel with a watch glass during filtration, and when sufficient filtrate has been collected, polarize in a 200 mm. polariscope tube at 20° C. In case the solution cannot be completely decolorized use a 100 mm. tube and multiply the reading by two.

Invert polarization.—Using the remainder of the clarified solution, precipitate the excess lead by carefully adding, either powdered anhydrous sodium oxalate or powdered anhydrous sodium carbonate, a small quantity at a time. Remove the precipitated lead by filtration through a dry filter paper, rejecting the first 15 cc.

Pipet 50 cc. of the clear lead-free filtrate into a 100 cc. volumetric flask. If sodium carbonate has been used to remove the lead, neutralize the excess with a few drops of dilute hydrochloric acid. Invert the sugar by either of the following methods:

(*a*) Add 25 cc. of distilled water and then while rotating, add gradually from a pipet 5 cc. of hydrochloric acid of sp. gr. 1.20. After the solutions have been mixed, heat the flask in a water bath at 70° C. The solution should reach a temperature of 67° C. in 2.5–3 minutes. Maintain the temperature as nearly as possible at 69° C. for 7–7.5 min-

utes making the total time of heating 10 minutes. Remove the flask and rapidly cool to 20° C. in running water; dilute to 100 cc. and shake.

(*b*) While rotating the flask, add gradually from a pipet 5 cc. of hydrochloric acid of sp. gr. 1.20. Set aside for 24 hours at a temperature not below 20° C. Cool to 20° C., dilute to 100 cc. and shake.

Polarize the inverted solution in a 200 mm. polariscope tube at 20° C. Because of the variation of the specific rotation of fructose with temperature, it is necessary to polarize at a constant definite temperature. For this purpose use a water-jacketed tube, circulating through it water at 20° C. Since the dilution of the solution is doubled after the direct polarization, multiply the reading for the invert polarization by two. Calculate the percent of sucrose by the following forumla:

$$S = \frac{100\,(P - I)}{142.66 - \frac{T}{2} - 0.0065\left[142.66 - \frac{T}{2} - (P - I)\right]}$$

which for 20° C. is simplified to:

$$S = \frac{100 - (P - I)}{(132.66 - 0.0065)\,[132.66 - (P - I)]}$$

where:

S = per cent of sucrose;
P = direct reading;
I = invert reading;
T = temperature at which the readings are made.

When the invert solution contains more than 13 grams of invert sugar per 100 cc., the following formula is used for the calculation of sucrose:

$$S = \frac{100\,(P - I)}{142.66 - \frac{T}{2}}$$

which for 20° C. is simplified:

$$S = \frac{100\mathrm{D}}{132.66} = 0.7358\mathrm{D}$$

Browne, C. A. Handbook of sugar analysis. John Wiley & Sons., Inc., New York.

*Official and Provisional Methods of Analysis. *U. S. Dept. Agr. Bur. Chem. Bull.*, 107, 39–41 (1912).

XII. Determination of Reducing Sugars

Expt. 139. Gravimetric Method.—Place a Gooch crucible in a holder, and pour in a small quantity of the prepared asbestos suspended in distilled water; apply gentle suction, adding from time to time small quantities of the asbestos, until a layer 1–1.5 cm. thick is obtained. Wash thoroughly with distilled water to remove fine particles of asbestos; then wash again using 10 cc. of redistilled ethyl alcohol, and finally, 10 cc. of ether. Suck dry. Remove the crucible, carefully wiping the outside, and dry for an hour in an oven at 100° C. Cool in a desiccator for 30 min. and then weigh.

The conditions under which the reduction is performed must be carefully standardized. The amounts of solutions used and the conditions of heating are those prescribed by Munson and Walker. Pipet 25 cc. each of Fehling's solutions A and B (Expt. 118) into a 400 cc. Pyrex beaker. Add 50 cc. or less of an approximately neutral sugar solution containing from 20 to 200 mg. of reducing sugar; but if less than 50 cc. is used, add sufficient distilled water to make the final volume 100 cc. Mix the solutions thoroughly and carry out the reduction. Cover the vessel with a watch glass and bring to the boiling point in exactly 4 minutes and then boil for exactly 2 minutes.

Cole describes an apparatus for the standardization of heating. This is accomplished by interposing a small manometer between the gas tap and the burner to obtain a constant gas pressure. It is illustrated in Fig. 25. The manometer tube contains a dilute solution of a colored substance, such as eosin. Add to a 400 cc. Pyrex beaker 25 cc. of each of the Fehling's solutions and 50 cc. of distilled water, and cover with a watch glass. Place a wire gauze having an asbestos center on a tripod and set the flask upon it. Turn on the gas tap to its full extent and light the burner, then tighten clamp, *A*, until the pressure is reduced about one-third. Observe the time required to raise the temperature to the boiling point. If boiling begins in less than 4 minutes, tighten clamp, *A*, and repeat the experiment until the standardizing solution does boil in exactly 4 minutes. Note and mark the reading of the manometer. This level should be maintained; therefore carefully observe it from time to time and adjust the clamp as the gas pressure varies.

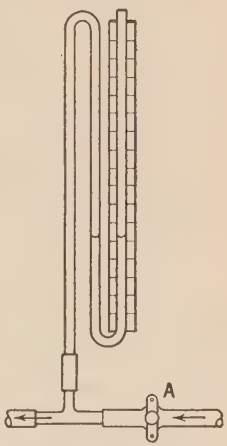

Fig. 25.

When the manometer has been properly adjusted, substitute the reducing sugar mixture for the standardizing solution and proceed with the reduction as described. When complete, at once filter the cuprous oxide on the asbestos layer of a Gooch crucible and wash thoroughly with distilled water at a temperature of about 60° C., then with 10 cc. of redistilled ethyl alcohol, and finally with 10 cc. of ether. Dry for 30 minutes in an oven at 100° C.; cool and weigh. Repeat this process until a constant weight is obtained. Make a *blank* determination using 50 cc. of the Fehling's solutions and 50 cc. of distilled water. If more than 0.5 mg. of cuprous oxide is obtained, subtract the result from the reducing sugar determination. The alkaline tartrate solution deteriorates on standing so the amount of cuprous oxide obtained increases with the age of the solution. By reference to the Munson and Walker table, determine the weight of sugar equivalent to the weight of cuprous oxide formed in the reduction.

After 8 or 10 determinations have been made with each Gooch crucible, dissolve the cuprous oxide in nitric acid; wash the asbestos felt thoroughly with distilled water and use over and over again for similar determinations. After completing the experiment return the washed asbestos to a bottle provided for this purpose.

Asbestos.—Digest the amphibole variety of asbestos with dilute hydrochloric acid (1 : 3) for three days. Wash free from acid on a large Büchner funnel, and again digest for an equal time with 10 per cent sodium hydroxide solution. Drain the asbestos on a Büchner funnel and treat for 2 or 3 hours with hot alkaline tartrate solution (Expt. 118). Then wash practically free from alkali and digest for several hours with dilute nitric acid (1 : 3). Wash free from acid and shake to a fine pulp with distilled water.

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Expt. 140. Volumetric Method.—For the determination of reducing sugars it is necessary to employ a method which is both rapid and

accurate. The application of the reduction of Fehling's solution is best suited for this purpose. Of the two methods possible, the gravimetric and volumetric, it has been clearly shown that where rapidity is of importance the volumetric method only should be considered. In either case after reduction the solution contains the oxidation products of the sugar, an excess of cupric salt, and suspended cuprous oxide. The amount of sugar oxidized may be learned, either by making a determination of the cuprous oxide formed, or, knowing the total amount of copper solution used, by the residual cupric salt. Both cupric and cuprous salts may be iodemetrically determined by means of the reversible reaction:

$$2CuSO_4 + 4KI \leftrightarrows 2CuI + I_2 + 2K_2SO_4$$

Shaffer and Hartmann have established the principles underlying these methods and the conditions necessary for the determination of both forms of copper.

" In one case, the residual cupric salt is completely converted into cuprous iodide, with the liberation of an equivalent amount of iodine.

$$2Cu^{++} + 4I^- \leftrightarrows 2CuI + I_2$$

In the other case cuprous salt is completely iodized to cupric in the presence of a known excess of iodine, with the conversion of the corresponding amount of iodine into iodide.

$$2Cu^{++} + 2I^- \leftarrow 2Cu^+ + I_2$$

The iodine formed from iodide in the first case, and the excess iodine left in the second case, are determined by titration with standard sodium thiosulfate, starch being used as indicator."

The direction in which the reaction takes place depends upon the concentration of the active substances or ions. For the complete conversion of cupric to cuprous salt, the concentration of the iodide must be relatively high; while for the oxidation of cuprous salts by iodine the concentration of cupric and iodide ions must be maintained at very low values. This can be accomplished by dilution, but it is not feasible. Elbs, however, has discovered another method of reducing the concentration of cupric ions to such a small value that in the presence of a large amount of soluble iodide the reaction does not proceed to the right, and equilibrium is maintained with copper entirely in the cupric form. He has shown that alkali oxalates inhibit the reaction between cupric salts and soluble iodides; consequently the addition of an alkali oxalate to the reduced solution shifts the equilibrium entirely to the cupric side of the reaction when in the presence of an excess of

potassium iodide, and the cuprous iodide is at once dissolved and oxidized by the free iodine. By taking advantage of the action of the oxalate there are two methods available for the iodometric determination of the amount of copper reduced by the sugar. After acidification of the alkaline copper solution either the residual cupric salt or the cuprous salt may be titrated with standard sodium thiosulfate, following their reaction with iodide or iodine respectively. Either the cupric or cuprous salt may be determined in the presence of the other. The cupric titration is essentially that introduced by Lehmann and more recently modified by Peters.

Reduction.—Pipet 25 cc. each of Fehling's solutions A and B (Expt. 118) into a 300 or 400 cc. wide-mouthed Erlenmeyer flask. Add 50 cc. or less of the approximately neutral sugar solution containing from 20 to 200 mg. of sugar and, if necessary, sufficient distilled water to make the final volume 100 cc. Mix the solutions thoroughly and carry out the reduction (Expt. 139). Place the flask immediately in running water until cool. This will require three or four minutes.

Cuprous titration.—The determination is based upon the reoxidation of the cuprous salt which is made possible by the action of the oxalate. Add from a calibrated pipet 50 cc. or 25 cc. of the iodate-iodide solution, depending upon the amount of cuprous oxide present. If the solution reduced, contains 100 or more milligrams of sugar, use 50 cc. of the iodate-iodide solution, but if it contains less use only 25 cc. of this solution. There must be present an excess of acid which is sufficient to dissolve the cuprous oxide and at the same time liberate the iodine from the iodate-iodide mixture according to the equation:

$$KIO_3 + 5KI + 3H_2SO_4 \rightarrow 3K_2SO_4 + 3I_2 + 3H_2O$$

A very great excess, however, causes the decomposition of the complex oxalate and must be avoided. Add 15–17 cc. of 5 *N* sulfuric acid from a fast-flowing pipet or cylinder in order to acidify the whole solution promptly. Rotate the flask to keep the solution of cuprous oxide in suspension during the addition of the acid. Organic acids are oxidized by hypoiodite in *alkaline* solution and those present may use some of the iodine if the sulfuric acid is added very slowly; therefore add it quickly. The solution should become clear, although often some cuprous iodide will separate. Add 20 cc. of saturated potassium oxalate solution and rotate the flask until the cuprous iodide is *completely* dissolved. However, if the alkaline solution is taken from the running water while the temperature is still over 40° C., and the iodate-iodide, acid, and oxalate are added, the cuprous oxide dissolves almost immediately and the solution remains clear. When the solution is cold the

cuprous iodide may first separate and when this is dissolved after the addition of the oxalate, acid potassium tartrate may crystallize, but neither affects the results. Titrate the solution with standard sodium thiosulfate, adding 3 cc. of soluble starch solution toward the end of the titration before the green color disappears. The starch blue end-point is remarkably sharp and distinct and need not be confused with the blue color due to the complex copper salt. If the solution is cold, a precipitate of cupric oxalate or cupric tartrate may separate toward the end of the titration, but neither will interfere.

The amount of iodine added in the iodate-iodide solution and the blank reduction of the Fehling's solution must be accurately known. Boil the Fehling's solution with 50 cc. of distilled water instead of the sugar solution, cool, and proceed as described above. The value of the blank remains practically unchanged for a long time and need be determined only occasionally. Subtract from the blank titration the titration of the sugar determination. The remainder represents the iodine required for the oxidation of the cuprous salt. Determine the equivalent in terms of metallic copper and find the amount of sugar equivalent to the copper by reference to Munson-Walker tables.

Sodium thiosulfate solution.—Make triplicate determinations. Prepare 2 liters of solution. Dissolve 51 gm. of sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, and 5 gm. of sodium hydroxide per liter of distilled water. The latter will neutralize the effects of any carbon dioxide present in the water. Preserve in an amber-colored bottle to exclude actinic light. A solution thus prepared and protected will keep its original strength almost indefinitely.

Standardization of the sodium thiosulfate solution.—Weigh accurately 100–150 mg. of pure copper foil which has been previously cleaned with dilute nitric acid to remove any coating of foreign material, and dried. Place it in a 300–400 cc. Erlenmeyer flask. Dissolve in 5 cc. of a 1 : 1 mixture of concentrated nitric acid and distilled water and heat on an asbestos gauze until brown fumes are no longer apparent. Then add 25–30 cc. of distilled water and a little powdered talcum and boil the mixture vigorously from five to ten minutes. Test the escaping vapors for nitrous acid with potassium iodide starch paper until the test becomes negative. This nitric-acid-talcum procedure effectively removes all the decomposition products of nitric acid that would interfere with the accuracy of the iodide method. Cool the solution to room temperature and dilute to 100 cc. with distilled water. Acidify the solution with an excess of sulfuric acid. For this purpose add 2.6 cc. of concentrated acid. Cool the solution to room temperature and add 5 cc. of a saturated solution of potassium iodide. Titrate at once with

the sodium thiosulfate solution until the brown tinge has become weak, then add about 3 cc. of the soluble starch solution or enough to produce a marked blue coloration. Continue the titration cautiously until the color due to free iodine has entirely vanished. Toward the end the blue color changes to a faint lilac. If at this point the thiosulfate be added drop by drop, there is no difficulty in determining the light-cream white end point within a single drop. However, if the solution stands a short time exposed to the air it again takes on a faint blue color. Determine the copper equivalent of the sodium thiosulfate solution.

Iodate-iodide solution.—Dissolve 5.4 gm. of potassium iodate and 60.0 gm. of potassium iodide in distilled water to which 1 gm. of potassium hydroxide has been added, and dilute to a liter. The alkali prevents the formation of hydriodic acid and its oxidation by the air.

Starch.—Mix about 0.5 gm. of soluble starch with cold distilled water to a thin paste; pour this into 100 cc. of boiling water and boil for a few minutes.

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Peters, A. W. A critical study of sugar analysis by copper reduction methods. *J. Am. Chem. Soc.*, 34, 928–954 (1912).

Elbs, K. Beispiel für umkehrbare Reaktion und Komplexsalzbildung. *Z. Electrochem.*, 23, 147–148 (1917).

*Methods of Analysis. Asso. Off. Agr. Chemists, 321–331. 1920.

*Shaffer, P. A., and Hartman, A. F. The iodometric determination of copper and its use in sugar analysis. I. Equilibria in the reaction between copper sulfate and potassium iodide. *J. Biol. Chem.*, 45, 349–364 (1921).

*Shaffer, P. A., and Hartmann, A. F. The iodometric determination of copper and its use in sugar analysis. II. Method for the determination of reducing sugars in blood, urine, milk, and other solutions. *J. Biol. Chem.*, 45, 365–390 (1921).

Myers, V. C., and Croll, Hilda M. The determination of carbohydrates in vegetable foods. *J. Biol. Chem.*, 46, 537–551 (1921). The picric acid colorometric method is used.

XIII. Sugars in Plant Tissue

Expt. 141. Preparation of Plant Tissue for Carbohydrate Studies.—According to Bourquelot, the plant tissue must be placed in boiling ethyl alcohol as soon as possible after collection in order to destroy the enzymes, which might otherwise change the carbohydrates present;

also precipitated calcium carbonate must be added to neutralize the plant acids and to prevent inversion of the compound sugars. Although calcium carbonate gives a slightly alkaline solution, this is not sufficient to cause enolization.

Preparation and extraction of the sample.—Determine the moisture content of the plant material. Calculate the volume of 95 per cent ethyl alcohol necessary to make a final concentration of 80 per cent when 100 gm. of plant tissue are used. Place this in a 750–1000 cc. Erlenmeyer flask and add also 1.5 gm. of pure precipitated calcium carbonate for each 100 gm. of plant tissue. Heat the contents of the flask nearly to boiling on an electric hot plate; then in the course of 10 minutes add the fresh tissue, a small quantity at a time. Boil the sample for half an hour under a reflux condenser; when partially cool, stopper the flask tightly with a rubber stopper and preserve until needed for the extraction of the sugars. Boiling alcohol of the above concentration destroys enzyme action, but does not necessarily destroy the action of all molds and bacteria. The extraction is carried out in a continuous extraction apparatus of the Soxhlet type as modified by Newton; it is illustrated in Fig. 26. This consists of a 750 cc. wide-mouthed bottle, *A*, having a small hole drilled near the bottom in which a glass siphon, *B*, is fitted, either by making a ground-glass joint or by wedging it with a collar of rubber tubing. The siphon is cut off even with the bottom of the bottle, which is covered with glass beads. The bottle is closed with a rubber stopper, carrying two bent tubes, each connected with a reflux Hopkins' condenser, and a distilling tube, *C*, which leads to the 2-liter short-necked flask, *D*. The efficiency of the distilling tube is increased by covering it with a jacket of rubber tubing. Siphon, *B*, is connected by rubber tubing with a glass tube in the stopper of flask, *D*. This tube has an air vent, *E*, which prevents premature siphoning by liquid collecting in the bottom of the tube. The flask is heated in a steam bath. Transfer the preserved sample to the extractor and extract with 80 per cent ethyl alcohol on a steam bath for about 30 hours, or until a sample of the alcohol in the extractor gives a negative test with α-naphthol (Expt. 114). The extraction is generally complete

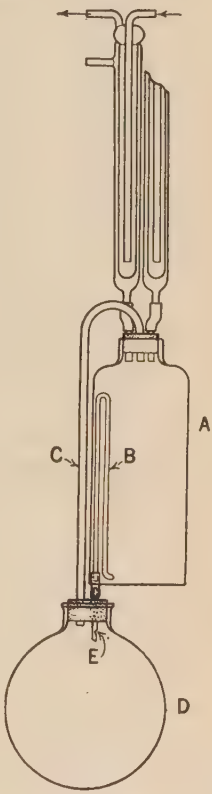

FIG. 26.

when the green tissue becomes colorless. Preserve the residue for the determination of pentosans (Expt. 151) and of starch (Expt. 157). Cool and filter the extract and thoroughly wash with 80 per cent alcohol. This extract contains the soluble sugars and soluble nitrogen. What sugars have been discovered in plant tissue?

Concentrate the extract in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 75–100 cc.; add 200 cc. of distilled water and again concentrate the solution. This process removes the alcohol, so that a determination of aliphatic amino nitrogen (Expt. 110) may be made. Filter the extract through four layers of cheese cloth on a small funnel into a 250 cc. calibrated volumetric flask; make nearly to volume by washing the distilling flask with several small portions of hot distilled water. The filtration removes the particles of chlorophyll which have separated. Cool the solution to room temperature and make to volume.

Clarification: Method I.—By this method, proposed by Horne, the error resulting from the volume of the precipitate is largely eliminated and it is not necessary to make the solution to volume again. Add to the solution dry powdered normal lead acetate; a small quantity at a time. After each addition, filter a small portion of the mixture through a *dry* filter paper and test for complete precipitation. When this results, filter the entire mixture through a dry filter paper in a large funnel; cover the funnel with a watch glass to prevent evaporation and collect the filtrate in a narrow-necked flask. Delead the filtrate by the addition of a minimum quantity of powdered potassium oxalate, testing small portions as before. Finally filter through a dry filter paper. If the sugar solution is not to be analyzed immediately, add toluene and shake well; this prevents any bacterial action or fermentation. This method of clarification should be used only when a small amount of impurity is present.

Method II.—If large quantities of impurities are present in the plant extract, the use of powdered normal lead acetate is not practicable and basic lead acetate solution should be substituted. Add to the extract basic lead acetate solution just sufficient to precipitate the impurities. Make frequent tests with small portions to determine this. Then filter, wash the precipitate several times with distilled water and *immediately* delead the filtrate by adding pulverized disodium phosphate until no more precipitate is produced. Filter into a 500 cc. volumetric flask, and make to volume with distilled water.

Basic lead acetate solution.—Mix 430 gm. of pulverized normal lead acetate, 130 gm. of litharge and 1000 cc. of distilled water, and boil under a reflux condenser for half an hour. Allow the mixture to cool

and settle, and dilute the supernatant liquid to a sp. gr. of 1.25 with recently boiled distilled water. If desired, 560 gm. of Horne's dry basic lead acetate may be substituted for the normal salt and the litharge.

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Kraus, E. J., and Kraybill, H. R. Vegetation and reproduction in the tomato. *Ore. Agr. Expt. Sta., Bull.*, 149 (1919).

*Newton, R. A comparative study of winter wheat varieties with especial reference to winter-killing. *J. Agr. Sci.*, 1–19. Cf. 7–9 (1922).

*Englis, D. T., and Tsang, C. Y. The clarification of solutions containing reducing sugars by basic lead acetate. The effect of different deleading agents. *J. Am. Chem. Soc.*, 44, 865–867 (1922). The authors find that disodium phosphate is the most satisfactory deleading agent.

Link, K. P., and Tottingham, W. E. Effect of the method of desiccation on the carbohydrates of plant tissue. *J. Am. Chem. Soc.*, 45, 439–447 (1923).

Hildreth, A. C., and Harvey, R. B. Grinding wood samples for analysis. *Botan. Gaz.*, 78, 460–461 (1924).

Sando, C. E. Continuous extraction apparatus for large quantities of plant materials. *Ind. Eng. Chem.*, 16, 1125 (1924).

Link, K. P. Effects of the method of desiccation on the carbohydrates of plant tissue. *J. Am. Chem. Soc.*, 47, 470–476 (1925).

Expt. 142. Identification of Unknown Sugars.—If plant tissue has been prepared for carbohydrate studies (Expt. 141), use a portion of this solution for the experiment; otherwise the instructor will furnish a different unknown mixture of two pure sugars to each student. The individual sugars are to be identified by the application of methods previously described in this chapter.

Browne, C. A. A handbook of sugar analysis. John Wiley & Sons, New York, 1912.

van der Haar, A. W. Anleitung zum Nachweis, zur Trennung und Bestimmung der reinen und aus Glukosiden u.s.w. erhaltenen Monosaccharide und Aldehydsäuren. Gebrüder Borntraeger, Berlin, 1920. For review by C. A. Browne, see *J. Am. Chem. Soc.*, 43, 685–687 (1921).

XIV. Preparation of Sugars

Certain general methods are used in the preparation of pure sugars. The raw material is ground fine and extracted with water as in the

preparation of inulin, or it may be hydrolyzed with dilute sulfuric acid, either under atmospheric pressure or in an autoclave. When the liquid is nearly boiling, it is neutralized to Congo red paper with barium carbonate and then filtered. In most cases, a small quantity of barium remains in the filtrate because of the solubility of the barium carbonate, though the combination of the latter with organic acids may also produce it. This should be removed by the addition of dilute sulfuric acid and by the subsequent filtration of the barium sulfate. The solutions obtained either by water extraction or by acid hydrolysis must be purified. For this purpose, normal or basic lead acetate and ethyl alcohol are most efficient; the nature of the impurities determines which shall be used. Either lead acetate produces a flocculent precipitate, which consists of proteins, tannins, organic acids, coloring matter, gum residues, and colloidal substances. This precipitate should be filtered off and the excess of lead acetate removed from the filtrate by the use of hydrogen sulfide, but the addition of enough to peptize the precipitated lead sulfide should be avoided. If the filtrate needs decolorizing, it should be done by adding to the cold solution a vegetable decolorizing carbon, such as Norit. The clear filtrate should then be concentrated under diminished pressure and poured into two or three volumes of ethyl alcohol. If any precipitate forms, it should be filtered off. After filtration the solution is again concentrated under diminished pressure to the desired percentage of total solids; this is determined by the use of an Abbé refractometer. The resulting sirup is poured into the required amount of glacial acetic acid or ethyl alcohol, and crystallization is allowed to take place. Both glacial acetic acid and ethyl alcohol are employed for crystallizing sugars, but the former, first used by Wernicke and Pfitzinger for crystallizing sucrose, is now preferred in most cases. The crystals are separated from the mother liquor by filtration on a Büchner funnel and are then dried. Each sugar should be standardized according to the methods of Graber. This analysis should include a determination of the moisture, specific rotation, and ash content.

Brewster's internally heated vacuum apparatus.—The sugar solution can be concentrated under diminished pressure either in a 1000 cc. Claisen distilling flask (p. 136), or in the internally heated vacuum apparatus described by Brewster and illustrated in Fig. 27. For less than two liters of solution the first apparatus is preferable; but with larger volumes, the latter should be used. The main portion of the apparatus consists of an inverted bell jar of approximately, 30 × 17 cm., having a ground flange. The jar is supported by being inserted through a hole of the same diameter in a board frame, until it

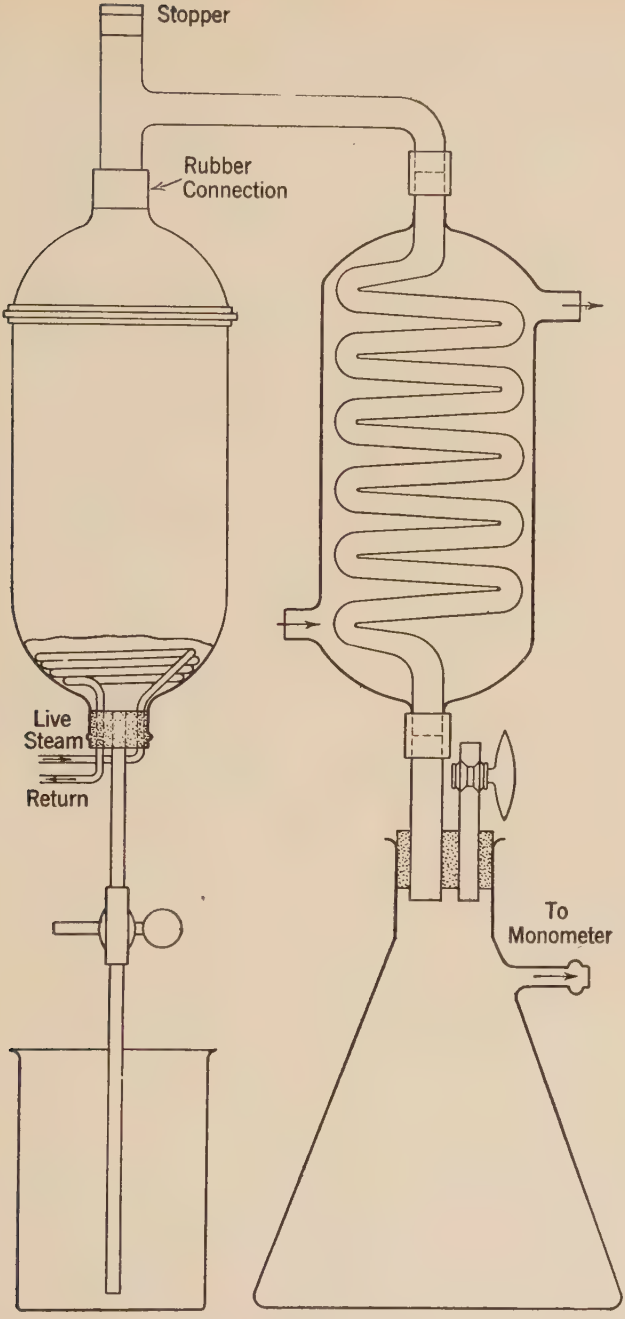

FIG. 27.

rests upon the flange. The tubulure is closed with a three-hole rubber stopper, carrying a glass tube which serves both as the feed and the discharge tube, and the two leads of the heating coil. The latter is of Monel metal tubing, 5 mm. in diameter, coiled close together two and a half times; then the ends are brought close to the center and bent at right angles to the spiral plane so that they may pass through the rubber stopper and connect, one with the source of heat, and the other with the drain. The bell jar is covered with the glass dome of an ordinary distilling apparatus, the flanges of both being ground so that they form a tight joint, otherwise a rubber gasket must be used. The tubulated top of a vacuum desiccator or a second bell jar may serve as the dome. The latter is especially useful in the concentration of a protein solution, which tends to froth. The tubulure of the dome is connected by rubber tubing with a T-tube, one arm of which is a delivery tube leading to a condenser, which in turn discharges into a 5-liter Erlenmeyer filtering flask. A long rigid thermometer may be inserted through the stopper in the top of the T-tube. An *efficient* condenser is essential to rapid distillation. For this purpose a closely wound helix of either block tin or copper tubing 12–19 mm. in diameter, surrounded by a water jacket, may be used. The filtering flask is attached to a manometer as illustrated in Fig. 27, p. 209.

During distillation the heating coil is connected with either live steam or an instantaneous water heater. A pressure of 30 mm. or less should be maintained and the temperature should be kept at 45–50° C., being controlled by varying the steam or water pressure.

Wernicke, A., and Pfitzinger, W. Method of extracting crystallizable sugar from molasses. U. S. Patent 260,340. Dated June 27, 1882.

Pfanstiehl, C., and Black, R.S. The rare sugars: their purity and tests. *J. Ind. Eng. Chem.*, 13, 685–687 (1921).

*Graber, H. T. The standardization of rare sugars. *J. Ind. Eng. Chem.*, 13, 687–688 (1921).

Browne, C. A. Moisture absorptive power of different sugars and carbohydrates under varying conditions of atmospheric humidity. *J. Ind. Eng. Chem.*, 14, 712–714 (1922).

*Brewster, J. F. An internally heated laboratory vacuum pan. *Ind. Eng. Chem.*, 15, 139 (1923).

Expt. 143. ***d*-Xylose from Corn Cobs.**—Corn (*Zea mays*) cob meal, which is rich in the pentosan, xylan, is the best source of material for the preparation of xylose. The meal may be hydrolyzed either under atmospheric pressure or in an autoclave according to the method of Fred and Peterson.

Hydrolysis.—Place 250 gm. of corn cob meal in a 4-liter Erlenmeyer flask; add 1.5 liters of 2 per cent, by weight, sulfuric acid, and heat in

an autoclave for 3 hours at 15 lbs. pressure. If an autoclave is not available, place the meal in a 4-liter round-bottomed Pyrex flask, add 1.5 liters of 4 per cent, by weight, sulfuric acid, and hydrolyze by boiling under a reflux condenser on a sand bath, for 3 hours. After completing the hydrolysis, filter the extract through 8 oz. cotton duck and press the residue as dry as possible with a hand press. Then wash the residue on a Büchner funnel with 400–500 cc. of distilled water.

Neutralization.—Heat the combined extract and wash water nearly to boiling, and neutralize by adding a slight excess of the calculated amount of precipitated barium carbonate. Continue to heat until the mixture tests neutral to Congo red paper. The heating serves a double purpose: first, it accelerates the reaction, which is necessarily slow on account of the slight solubility of the barium carbonate; second, it causes the digestion of the barium sulfate so that it is more easily filtered and washed. Filter off the barium sulfate at once on a Büchner funnel and wash with hot distilled water. A small quantity of barium remains in the filtrate because of the solubility of the barium carbonate, though the combination of the latter with organic acids may also produce it. To remove the excess of barium, carefully add dilute sulfuric acid (1 : 20) until no further precipitate of barium sulfate is formed. At this stage the solution should not change the color of Congo red paper. Filter off the barium sulfate.

Purification.—Acidify the filtrate with a few cubic centimeters of glacial acetic acid and decolorize, while cold, with the vegetable decolorizing carbon, Norit, in the ratio of 20 gm. per liter of xylose solution. Filter and concentrate the solution in a 1000 cc. Claisen distilling flask under diminished pressure (p.136) to 60–65 per cent solids, as determined by the Abbé refractometer (Expt. 67). Pour the sirup into 3 volumes of 95 per cent ethyl alcohol, which is stirred constantly; allow to stand over night until the precipitate separates; then filter. This treatment reduces the percentage of impurity in the sugar.

Crystallization.—Again concentrate the filtrate under diminished pressure to 75–80 per cent solids. Add an equal volume of glacial acetic acid to the flask; warm and shake until the contents are thoroughly mixed; pour into a beaker and allow to cool. *Seed* with a few crystals of xylose and place in a refrigerator over night for crystallization to begin; then freeze in a mixture of ice and salt, stirring as long as possible. Replace the frozen mass in the refrigerator where it will thaw out slowly. A week is usually necessary for complete crystallization. Filter off the crystals on a Büchner funnel, fitted with a hardened filter paper, sucking as dry as possible, and finally using the rubber pressure device (Expt. 101). Return the crystalline mass to the beaker, mix with sufficient

glacial acetic acid to form a thick paste and again filter. Repeat this operation with glacial acetic acid, several times with 95 per cent ethyl alcohol until small portions of the filtrate show no acid test to litmus, and finally, once with absolute ethyl alcohol. Dry in the air carefully protected from dust, and finally, either in a vacuum desiccator over sulfuric acid for several days, or in a vacuum oven at 70° C. for 3 or 4 hours. The yield of *d*-xylose is about 12 per cent of the weight of cobs used. Determine the ash content. Also determine both the initial and final specific rotations (Expt. 136) in water at 20° C. For this purpose, dissolve 4 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

Stone, W. E., and Lotz, D. A new source for xylose. *Am. Chem. J.*, 13, 348–350 (1891). These authors were the first to use corn cobs for the preparation of xylose.

Hudson, C. S., and Harding, T. S. The preparation of xylose. *J. Am. Chem. Soc.*, 39, 1038–1040 (1917). Cottonseed hulls were the source of material.

*Hudson, C. S., and Harding, T. S. The preparation of xylose from corn cobs. *J. Am. Chem. Soc.*, 40, 1601–1602 (1918).

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*Harding, T. S. The sources of the rare sugars. III. History of xylose, its discovery and methods of preparation. *Sugar*, 25, 124–125 (1923).

Hirst, E. L., and Purves, C. B. The structure of the normal monosaccharides. Part I. Xylose. *J. Chem. Soc.*, 123, 1352–1360 (1923).

Expt. 144. *l*-Arabinose from Mesquite Gum.—According to Ehrlich, the pectin of plants consists of calcium-magnesium salt of a complex anhydro-arabino-galactose-methoxyl-tetragalacturonic acid. The exact combination of the units in the pectin is uncertain but it is known that, upon hydrolysis, arabinose is easily split off while galactose is not. Hence, any material rich in pectin would be suitable for the preparation of this sugar.

Hydrolysis.—Dissolve 500 gm. of mesquite gum [5] in 3 liters of water

[5] Mesquite gum is collected by the Indians and Mexicans of the southwestern U. S. and northern Mexico. It is carried by most of the drug stores of Tucson and with a few weeks' notice could be supplied in large amounts by the Martin Drug Company of Tucson, Arizona.

contained in a 5-liter flask, by allowing the gum and water to stand over night or by heating for an hour in a boiling water bath. To the cold solution add a cool solution of 125 gm. of concentrated sulfuric acid in 70 cc. of water, and heat to 80° C. for 3 hours in a large water bath.

Neutralization.—Precipitate the sulfuric acid by adding to the hot solution, a hot concentrated solution of 410 gm. of crystalline carbonate-free barium hydroxide. Adjust the solution to neutrality by adding small amounts of barium hydroxide solution or sulfuric acid solution as the case may require. Allow the barium sulfate to settle; siphon the clear solution; filter the barium sulfate on a Büchner funnel, and wash it with hot water. Combine the filtrate and the washings and concentrate under reduced pressure (p. 136) to a volume of approximately 650 cc. Transfer the sirup to a 3 liter flask, using not more than 50 cc. of wash water.

Purification.—To the resulting solution, add with shaking, 1.5 liters of 95 per cent ethyl alcohol. Allow to stand until the solution is no longer turbid. Decant the sugar solution from the gummy barium salts and extract the latter four times under the reflux condenser, using 500 cc. of boiling methyl alcohol for each extraction. After the fourth extraction, transfer the salt to an evaporating dish and grind to a powder. Concentrate the ethyl alcohol solution of the sugars *in vacuo* on the boiling water bath to 40–50 per cent solids and cool. After crystallization has begun add a small volume of ethyl alcohol, being careful not to precipitate an appreciable amount of gummy material. Set the solution in the refrigerator until crystallization is complete. Filter off the crystals, wash with 95 per cent alcohol and dry. Combine the filtrate with the methyl alcohol extract of the barium salts, shake, allow to settle, pour off the supernatant liquid and concentrate it *in vacuo*. To secure a second crop of crystals repeat the process. The filtrate from the first two crops of crystals contains gummy materials which frequently interfere with the crystallization of the sugar. To avoid this, concentrate the combined filtrates to a very thick sirup by distillation *in vacuo* on the boiling water bath. Dissolve the residue in a small volume of hot methyl alcohol. Precipitate the gums by the addition of a limited amount of 95 per cent ethyl alcohol. This also precipitates a small amount of sugar; therefore an excess of alcohol should be avoided. Decant the sugar solution and concentrate by distillation *in vacuo* on the boiling water bath. Again dissolve the sugar in a small volume of hot methyl alcohol, cool, seed with pure arabinose, and place in the refrigerator to crystallize. Filter off the crystals and repeat the process twice.

Crystallization.—Dissolve the crystals of *l*-Arabinose in eight times

their weight of hot water, decolorize with the vegetable decolorizing carbon, Norit, by heating the solution to gentle boiling for half an hour. Filter the hot solution and concentrate the filtrate *in vacuo* on the boiling water bath to 70–75 per cent solids. Transfer the hot solution to a beaker, rinsing the flask with enough hot water to make the total volume in cubic centimeters the same as the original weight of the sugar in grams. Allow to cool, stirring occasionally. When cold, add 20 cc. of 95 per cent ethyl alcohol for every 100 gm. of *l*-arabinose, mix, and filter off the crystals. Again transfer them to the beaker, triturate with 30 cc. of ethyl alcohol for every 100 gm. of sugar, filter, and dry the crystals for several days in a vacuum desiccator over sulfuric acid. When the melting point of the original sugar is above 140° C. the yield of the first crop of purified crystals will be 70 per cent of the sugar used. The product should be perfectly white and should melt at 148–151° C. Concentrate the filtrate from the first crop and secure a second crop of less pure material. By crystallizing the purified material twice from a mixture of its weight of water and four times its weight of 95 per cent ethyl alcohol, a product melting at 156–160° C. is obtained.

Determine the ash content. Also determine both the initial and final specific rotation (Expt. 136) in water at 20° C. For this purpose dissolve 2 gm. in distilled water in a 50 cc. volumetric flask; make to volume and polarize in a 200 mm. polariscope tube.

Kiliani, H. Ueber Arabinose. *Ber.*, 19, 3029–3036. Cf. 3030 (1886). This method of preparing arabinose from cherry gum is among the first ever published.

von Lippman, E. O. Gummiartige Ausschwitzung an Zuckerrüben. *Ber.*, 23, 3564–3566 (1890). Furfuraldehyde is prepared from a gum exuding from sugar beets and the author concludes that arabinose could also be made from them.

Allen, E. W., und Tollens, B. Ueber Holzzucker (Xylose) und Holzgummi (Xylan.) *Ann.*, 260, 289–306. Cf. 298–301 (1890).

Ullik, F. Ueber Araban, ein der Arabinose entsprechendes Kohlenhydrat. *Oesterrung. Z. Zuckerind. Landw.*, 23, 268–275 (1894). The author prepared araban from beet pulp and from this he made arabinose.

Stone, W. E. The carbohydrates of the gum of *Acacia decurrens*. *Am. Chem. J.*, 17, 196–199 (1895). The author finds that this gum, like gum arabic and peach gum, yields both arabinose and galactose on hydrolysis.

Schindelmeiser, J. Ueber Arabinose in Weidengallen. *Sitzber. Natur. Gesell. Univ. Dorpat*, 15, 239–240 (1906). After extraction of tannin with a mixture of three parts ether and one part absolute alcohol, the residue was further extracted with alcohol. This removed the sugar, which was identified as arabinose. This indicates the existence of free arabinose in nature.

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Ehrlich, F. Die Pektinstoffe, ihre Konstitution und Bedeutung. *Chem. Ztg.*, 41, 197–200 (1917).

Harding, T. S. The sources of the rare sugars. *Sugar*, 24, 656–657 (1922). A discussion of the discovery and production of arabinose.

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Hirst, E. L., and Robertson, G. J. The constituion of the normal monosaccharides. Part II. Arabinose. *J. Chem. Soc.*, 127, 358–364 (1925).

Anderson, E., Sands, Lila, and Sturgis, N. Some plant gums of the southwestern United States. *Am. J. Pharm.*, 97, 589–592 (1925).

*Anderson, E., and Sands, Lila. Preparation of *l*-arabinose from mesquite gum. *Ind. Eng. Chem.*, 17, 1257–1258 (1925).

Expt. 145. *l*-Rhamnose from Quercitrin.—The glucoside, quercitrin, which occurs in the bark of the black oak (*Quercus coccinea*, var. *tinctoria* Gray), is the best source of material for the preparation of rhamnose. In 1854 Rigaud prepared quercitrin by extracting black oak bark with 85 per cent alcohol. He then hydrolyzed this compound with sulfuric acid, filtered off the quercetin, neutralized the filtrate with barium carbonate, concentrated the solution, and decolorized it with bone black. Crystals of rhamnose separated in five or six days. Although he did not name the sugar, this is apparently the first time it was prepared in crystalline form. Lemon Flavin, a commercial product made from the powdered bark, and rich in quercitrin, may be used for the preparation of rhamnose.

Hydrolysis.—Place 200 gm. of Lemon Flavin [6] in a 5-liter round-bottomed Pyrex flask; add 2 liters of 0.6 per cent, by weight, sulfuric acid, and boil under a reflux condenser on a sand bath for 1.5 hours. By hydrolysis, quercitrin yields rhamnose and the flavonol pigment, quercetin. The latter, when formed in the presence of water, shows marked imbibitional properties; hence the mixture becomes thick and at the same time changes to a color more intensely yellow than that of the original material. After hydrolysis, allow the mixture to stand over night, when the quercetin is practically insoluble. Filter on a large Büchner funnel, sucking as dry as possible, and finally using the rubber pressure device (Expt. 101). Return the residue to a beaker or flask, and boil with 750–1000 cc. of distilled water. Cool and filter.

Neutralization.—Heat the combined filtrate and wash water nearly to boiling, and neutralize by adding a slight excess of the calculated amount of precipitated barium carbonate. Continue to heat until the mixture tests neutral to Congo red paper. Filter off the barium sulfate at once on a Büchner funnel and wash with hot distilled water.

Purification.—Acidify the filtrate with a few cubic centimeters of

[6] This may be obtained from J. S. Young and Company, Hanover, Pa. There is no standard content of quercitrin in Lemon Flavin, consequently different samples may yield varying amounts of rhamnose.

glacial acetic acid and decolorize, while cold, with 15–20 gm. of the vegetable decolorizing carbon, Norit. Filter and concentrate the colorless solution in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 40 per cent solids as determined by the Abbé refractometer (Expt. 67). Pour the sirup into 3 volumes of 95 per cent ethyl alcohol, which is stirred constantly; allow to stand over night until the impurities separate; then filter. This treatment reduces the percentage of ash in the sugar.

Crystallization.—Again concentrate the filtrate under diminished pressure to 70–80 per cent solids. Immediately pour the colorless sirup into a beaker; place in the flask a quantity of glacial acetic acid equal to half the volume of the sirup; warm and shake until the contents are well mixed, and then add to the sirup in the beaker, with thorough stirring. Allow to stand in the refrigerator for two days, so that crystallization may take place. Filter the crystals on a Büchner funnel, fitted with a hardened filter paper, and drain as dry as possible, finally using the rubber pressure device. Return the crystalline mass to the beaker, mix with sufficient glacial acetic acid to form a thick paste and again filter. Repeat this operation several times, using absolute ethyl alcohol, until small portions of the filtrate show no acid test to litmus. Dry in the air carefully protected from dust and finally, either in a vacuum desiccator over sulfuric acid for several days, or in a vacuum oven at 30° C. for 3 or 4 hours. This sugar crystallizes with one molecule of water of crystallization, which it loses at 92.8° C. The yield of *l*-rhamnose is 10-25 per cent of the weight of Lemon Flavin used. Determine the ash content. Also determine the final specific rotation (Expt. 136) in water at 20° C. For this purpose, dissolve 3.5 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

*Rigaud, L. Ueber das Quercitrin. *Ann. Chem. Pharm.*, 90, 283–297. Cf. 295 (1854).

Votoček, E., und Frič, V. Berichte aus der Versuchsstation für Zuckerindustrie in Prag: XLVIII. Die Zuckerbestandtheile des Xanthorhamnins und Quercitrins. *Z. Zuckerind. Böhmen*, 25, 1–7 (1900–01). The authors demonstrated that xanthorhamnin yields on hydrolysis both galactose and rhamnose while quercitrin yields only rhamnose.

Clark, E. P. Preparation of rhamnose. *J. Biol. Chem.*, 38, 255–256 (1919).

*Walton, C. F., Jr. The preparation of rhamnose. *J. Am. Chem. Soc.*, 43, 127–131 (1921).

Harding, T. S. The sources of the rare sugars. II—History of rhamnose, its discovery and methods of preparation. *Sugar*, 25, 23–24 (1923).

Expt. 146. **β-*d*-Mannose from Vegetable Ivory Nut.**—The waste sawdust and turnings from vegetable ivory button factories are the best

source of material for the preparation of mannose. Vegetable ivory is the endosperm of the seed of the tagua palm (*Phytelepas macrocarpa*); on acid hydrolysis it yields a large proportion of mannose.

Extraction.—Heat 3 liters of 1 per cent sodium hydroxide solution to boiling in a porcelain evaporating dish or other suitable container; stir into it 300 gm. of vegetable ivory nut meal,[7] which has been passed through a sieve having 20 meshes to the linear inch. Remove the mixture at once from the source of heat and stir frequently during half an hour. Filter off the dark brown extract through heavy muslin or 8 oz. cotton duck and wash the residue with tap water until the filtrate is clear and neutral. Transfer the extracted residue to a tray and dry in an oven at a temperature not above 60° C.

Hydrolysis.—Place 165 cc. of cold 65 per cent, by weight, sulfuric acid in a 600 cc. Pyrex beaker, and add slowly, with constant stirring, 200 gm. of the thoroughly dried extracted meal. Do not permit the temperature of the mixture to rise above 50° C. Allow the brownish, dough-like material to stand over night to give time for the reaction to take place. Then dissolve it in 2 liters of distilled water; transfer to a 5-liter round-bottomed Pyrex flask, and boil under a reflux condenser on a sand bath for 3 hours.

Neutralization.—Heat the hydrolysate nearly to boiling, and neutralize it by adding a slight excess of the calculated amount of precipitated barium carbonate. Continue to heat until the mixture tests neutral to Congo red paper. The heating serves a double purpose: first, it accelerates the reaction, which is necessarily slow on account of the slight solubility of the barium carbonate; second, it causes the digestion of the barium sulfate so that it is more easily filtered and washed. Filter off the barium sulfate at once on a Büchner funnel and wash with hot distilled water. A small quantity of barium remains in the filtrate because of the solubility of the barium carbonate, though the combination of the latter with organic acids may also produce it. To remove the excess of barium, carefully add dilute sulfuric acid (1 : 20) until no further precipitate of barium sulfate is formed. At this stage the solution should not change the color of Congo red paper. Filter off the barium sulfate.

Purification.—Acidify the filtrate with a few cubic centimeters of glacial acetic acid and decolorize, while cold, with about 10 gm. of the vegetable decolorizing carbon, Norit. Filter and concentrate the colorless solution in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 60–65 per cent solids as determined by the Abbé

[7] The material may be obtained from the Janeway Button Company, New Brunswick, N. J.

refractometer (Expt. 67). Pour the sirup into 2 volumes of 95 per cent ethyl alcohol, which is stirred constantly; allow to stand over night until the precipitate separates; then filter. This treatment reduces the percentage of impurity in the sugar.

Crystallization.—Again concentrate the filtrate under diminished pressure to 87–88 per cent solids. Test a portion of the thick sirup for ketoses, using the Pinoff and resorcinol tests. Add an equal volume of glacial acetic acid to the flask; warm and shake until the contents are thoroughly mixed; pour into a beaker and allow to cool. *Seed* with a few crystals of mannose and place in a refrigerator over night for crystallization to begin; then freeze in a mixture of ice and salt, stirring as long as possible. Replace the frozen mass in the refrigerator where it will thaw out slowly. A week is usually necessary for complete crystallization, though the greater part of the sugar will crystallize in a day. Filter the crystals on a Büchner funnel, fitted with a hardened filter paper; and drain as dry as possible, finally using the rubber pressure device (Expt. 101). Return the crystalline mass to the beaker, mix with sufficient glacial acetic acid to form a thick paste and again filter. Repeat this operation with glacial acetic acid, several times with 95 per cent ethyl alcohol until small portions of the filtrate show no acid test to litmus, and finally, once with absolute ethyl alcohol. Dry in the air carefully protected from dust, and finally, either in a vacuum desiccator over sulfuric acid for several days, or in a vacuum oven at 70° C. for 3 or 4 hours. The yield of β-*d*-mannose is 42–45 per cent of the weight of extracted meal used. Determine the ash content. Also determine both the initial and final specific rotations (Expt. 136) in water at 20° C. For this purpose, dissolve 4 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

Fischer, E., und Hirschberger, J. Über mannose. I. *Ber.*, 21, 1805–1809 (1888).

Fischer, E., und Hirschberger, J. Über mannose. IV. *Ber.*, 22, 3218–3224 (1889). Mannose was separated as the hydrazone. This was purified by recrystallization and the mannose regenerated from it by hydrochloric acid.

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Expt. 147. ***d*-Galactose from Western Larch.**—A large number of galactans have been found in nature but only a few have been well characterized. Upon hydrolysis, many of them yield other sugars besides galactose, so that the terms, galacto-xylan, galacto-araban, galacto-mannan, etc., indicate the sugars obtained. In the western larch (*Larix occidentalis*), there is found a water-soluble galactan designated as ε-galactan, which upon acid hydrolysis yields only galactose.

Extraction.—Place 500 gm. of sawdust of the western larch, dried at about 65° C., in a 4-liter Erlenmeyer Pyrex flask; add 2.5 liters of distilled water, and boil under a reflux condenser on an electric hot plate for about 24 hours. Shake frequently during the operation. Filter off the amber-colored extract through several thicknesses of cheese cloth, by placing this over the mouth of the flask. Extract the residue with 1.5 liters of distilled water, heating as before for 24 hours. Extract a third time, heating 5 or 6 hours. Filter the combined extracts through filter paper, and concentrate the filtrate to 2 liters by evaporation on the steam bath.

Hydrolysis.—Place the galactan solution in a 4-liter Erlenmeyer flask; add sufficient sulfuric acid to make the solution 2.5 per cent, by weight, with respect to the acid, and boil under a reflux condenser for exactly 10 hours. Schorger and Smith found that a 1 per cent solution of galactan reaches maximum hydrolysis in 8 hours, and that thereafter the yield of galactose decreases.

Neutralization.—Heat the hydrolysate nearly to boiling, and neutralize it by adding a slight excess of the calculated amount of precipitated barium carbonate. Continue to heat until the mixture tests neutral to Congo red paper. The heating serves a double purpose: first, it accelerates the reaction, which is necessarily slow on account of the slight solubility of the barium carbonate; second, it causes the digestion of the barium sulfate so that it is more easily filtered and washed. Filter off the barium sulfate at once on a Büchner funnel and wash with hot distilled water. A small quantity of barium remains in the filtrate because of the solubility of the barium carbonate, though

the combination of the latter with organic acids may also produce it. To remove the excess of barium, carefully add dilute sulfuric acid (1:20) until no further precipitate of barium sulfate is formed. At this stage the solution should not change the color of Congo red paper. Filter off the barium sulfate.

Purification.—To the filtrate, add basic lead acetate solution (Expt. 141) until precipitation is complete. Filter over night on large funnels, fitted with fluted filter papers; and then wash with distilled water. Remove the excess of lead with pure hydrogen sulfide, taking care not to add enough to peptize the precipitated lead sulfide; then filter. A light straw-colored solution results.

Crystallization.—Concentrate the solution in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 75–77 per cent solids, as determined by the Abbé refractometer (Expt. 67). Add an equal volume of glacial acetic acid to the flask, shake until the contents are thoroughly mixed, and pour into a beaker. Rinse out the flask, using a volume of glacial acetic acid equal to that previously used, and add this to the mixture in the beaker, stirring thoroughly. Crystals begin to form in a few minutes. Allow to stand in the refrigerator for 2 or 3 days, when crystallization should be complete. Filter the crystals on a Büchner funnel, fitted with a hardened filter paper; and drain as dry as possible, finally using the rubber pressure device (Expt. 101). Return the crystalline mass to the beaker, mix with sufficient glacial acetic acid to form a thick paste and again filter. Repeat this operation with glacial acetic acid, several times with 95 per cent ethyl alcohol until small portions of the filtrate show no acid test to litmus, and finally, once with absolute ethyl alcohol. Dry in the air carefully protected from dust and finally, either in a vacuum desiccator over sulfuric acid for several days, or in a vacuum oven at 70° C. for 3 or 4 hours. The yield of *d*-galactose is 15–18 per cent of the weight of sawdust used. Determine the ash content. Also determine both the initial and final specific rotations (Expt. 136) in water at 20° C. For this purpose, dissolve 2 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

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*SCHORGER, A. W., and SMITH, D. F. The galactan of *Larix occidentalis*. *J. Ind. Eng. Chem.*, 8, 494–499 (1916).

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PRYDE, J. Constitutional studies in the monocarboxylic acids derived from sugars. Part I. Tetramethyl galactonolactone and the structure of galactose. *J. Chem. Soc.*, 123, 1808–1815 (1923).

Expt. 148. Purification of Sucrose.—Since sucrose is almost insoluble in absolute ethyl alcohol, its purification is usually carried out with alcohol as a precipitant. The solubility of sucrose in alcohol-water mixtures decreases very rapidly as the concentration of alcohol increases.

Dissolve 500 gm. of sucrose (granulated sugar) in 500 cc. of distilled water. Add 5–10 cc. of alumina cream (Expt. 64), mix thoroughly and filter on hardened fluted filter paper of large size. This treatment removes any suspended protein material or other impurities which have escaped clarification in the commercial refining process. Concentrate the water-clear filtrate at 40–45° C. in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 70–73 per cent solids as determined by the Abbé refractometer (Expt. 67). Pour the sirup into a beaker and add an equal volume of *pure* ethyl alcohol (Expt. 111), stirring meanwhile. Place in a refrigerator over night. Next day, if crystallization has not begun, *seed* with a few crystals of pure sucrose and replace in the refrigerator for several days. Centrifuge the crystalline mass or filter on a Büchner funnel, fitted with a hardened filter paper; drain as dry as possible, and finally use the rubber pressure device (Expt. 101). Return the crystalline mass to the beaker, mix with sufficient pure ethyl alcohol to form a thick paste and again centrifuge or filter. Repeat this operation with ethyl alcohol two or three times. Dry in the air on a glass plate carefully protected from dust, and finally in a vacuum desiccator over quicklime for several days, or use a vacuum oven at 70° C. for 3 or 4 hours. Determine the ash content. Also determine the specific rotation (Expt. 136) in water at 20° C. For this purpose, dissolve 3.25 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

Carefully test the sucrose for *reducing substances*. An ordinary Fehling's solution is unsuitable for the test, because of the destructive action of the free alkali upon the sucrose. In Striegler's solution, alkaline carbonates are used because of their relatively slight destructive action upon sucrose and high sensitivity toward invert sugar in small quantities. Heat 50 cc. of Striegler's solution to boiling and boil for one minute. Add 10 gm. of sucrose dissolved in 50 cc. of distilled water and boil the mixture for exactly 5 minutes. At the end of this period, stop the reaction by the addition of 100 cc. of cold, recently boiled distilled water; then filter on a previously weighed Gooch crucible as described in the determination of reducing sugars (Expt. 139). The

use of Striegler's solution causes the formation of a precipitate of not more than 0.007 gm. of cuprous oxide. If more is produced, the sucrose contains reducing substances.

Striegler's solution.—Dissolve 125 gm. of potassium bicarbonate, 50.7 gm. of potassium carbonate, and 3.464 gm. of cupric sulfate in distilled water and dilute to 500 cc. The solution is unstable on long standing.

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COHEN, E., und Commelin, J. W. Osmotische Untersuchungen. *Z. physik. Chem.*, 64, 1–52. Cf. 29 (1908).

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*POLARIMETRY. *Bur. Standards, Circ.*, 44. P. 99, 1918.

XV. SUGAR ALCOHOLS

Expt. 149. ***d*-Mannitol from Manna.**—Mannitol is a constituent of manna, which is a sweet exudation product of the European flowering ash (*Fraxinus ornus* Linne). Place 100 gm. of either large or small flake manna [8] in a 3-liter round-bottomed flask, add 500 cc. of 90 per cent ethyl alcohol and boil gently under a reflux condenser on a sand bath for one hour. Decant the hot supernatant liquid and, if necessary, filter through a hot-water jacketed funnel; then extract the residue again, using 250 cc. of 90 per cent ethyl alcohol. Allow the combined filtrates to stand in the refrigerator over night. Filter off the crystals on a Büchner funnel, fitted with a hardened filter paper; drain as dry as possible, finally using the rubber pressure device (Expt. 101). Dissolve the mannitol in the smallest possible quantity of boiling 80 per cent ethyl alcohol, decolorize with a few grams of the vegetable decolorizing carbon, Norit, filter and allow crystallization to take place in the refrigerator. Recrystallize once more. Filter and dry at 100° C. Determine the ash content. Determine the melting-point (Expt. 128). This should be 166.5° C. The mannitol should be free from reducing sugars. Test it by using Fehling's solution (Expt. 118). The aqueous solution of mannitol is optically inactive, but in the presence of borax its specific rotation is +22.5° at 20° C. To determine its specific rotation (Expt. 136) dissolve 5 gm. of mannitol and 6.4 gm. of crystallized borax in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C. and polarize in a 200 mm. polariscope tube.

[8] This material may be obtained from wholesale druggists.

Acetylation.—Place 1 gm. of mannitol in a large *dry* test tube, add a particle of fused zinc chloride half the size of a pea, and 5 cc. of acetic anhydride. Carefully heat the mixture over a free flame, shaking to get the materials into solution and to start the reaction. Remove the tube from the flame as soon as the reaction begins, for the heat generated by the latter is usually sufficient to carry it through. However, to make sure that the reaction is complete, warm the tube again and finally heat to boiling for half a minute; and then allow to cool. Add to the tube 25–30 cc. of distilled water and shake thoroughly to dissolve the excess of zinc chloride and acetic anhydride. The hexacetylmannitol separates as a sirup which soon becomes crystalline. Filter off the crystals, wash with distilled water and dry in air. Recrystallize from hot absolute ethyl alcohol. Dry the crystals at 100° C. and determine the melting-point (Expt. 128). This should be 110° C.

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Cake, W. E. The catalytic hydrogenation of dextro glucose. Preliminary notice. *J. Am. Chem. Soc.*, 44, 859–861 (1922).

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POLYSACCHARIDES

XVI. Pentosans

Expt. 150. Detection of Pentosans in Plant Material.—Pentosans are found in almost any plant tissue, either dried or fresh. Extraction is made with ethyl alcohol in order to remove pentoses, other sugars and glucosides; this is indicated, in the case of green material, by the practical disappearance of the green color. Because of the moisture in the fresh tissue, 95 per cent ethyl alcohol should be used for the extraction, and 85 per cent for dry material. Place 2–3 gm. of alfalfa (*Medicago sativa*) leaf meal in an Erlenmeyer flask, add 50 cc. of 85 per cent ethyl alcohol, attach a reflux condenser, heat in a water bath from 10 to 15 minutes, and filter. Repeat the extraction until the green color is practically removed. Dry the extracted residue and, if necessary, grind to a powder. Place 0.5 gm. of the pulverized material in a test tube, add 10 cc. of 12 per cent hydrochloric acid; boil the mixture from 3 to 5 minutes, and filter. Test the filtrate for the presence of pentoses (Expt. 123*b*). Also use a portion of the extracted material and perform the aniline hydrochloride test (Expt. 125).

Expt. 151. Quantitative Determination of Pentoses and Pentosans.—Place 3 gm. of alfalfa leaf meal (Expt. 150) in a 300 cc. distilling flask together with 100 cc. of 12 per cent hydrochloric acid, sp. gr. 1.06, and a few pieces of porous plate. Fit into the neck of the distilling flask a one-hole rubber stopper in which a small separatory funnel has been inserted. Connect the flask to a condenser and heat, gently at first, later regulating the flame so that about 30 cc. distills over in 10 minutes. Pass the distillate through a filter paper and catch it in a 500 cc. flask. After the distillation of each 30 cc. portion, add through the separatory funnel 30 cc. of 12 per cent hydrochloric acid, washing down the particles adhering to the sides of the flask. Continue the distillation until the distillate amounts to 360 cc. To the distillate add gradually, with stirring, 10 cc. of a 2 per cent solution of phloroglucinol in 12 per cent hydrochloric acid. Make the total volume to 400 cc. with 12 per cent hydrochloric acid and allow to stand over night.

Filter the black amorphous furfural phloroglucid in a weighed Gooch crucible (Expt. 139), and wash with 150 cc. of distilled water so that the asbestos mat is covered until all of the water has been added. Dry the crucible for 4 hours at 100° C. and weigh. From the increase in weight calculate the amount of pentoses or pentosans according to Kröber's tables or the formulae:

(1) When the weight of furfural phloroglucid (a) is less than 0.03 gm.

$$\text{Pentoses} = (a + 0.0052) \times 1.0170$$
$$\text{Pentosans} = (a + 0.0052) \times 0.8049$$

(2) When (a) is between 0.03–0.30 gm.

$$\text{Pentoses} = (a + 0.0052) \times 1.0075$$
$$\text{Pentosans} = (a + 0.0052) \times 0.8866$$

The factor 0.0052 is a correction for the weight of furfural phloroglucid remaining in solution in the 400 cc. of acid solution.

For advanced students interested in pentosan problems, the method developed by Pervier and Gortner, involving the electrometric titration of the furfural with bromine, is recommended.

*Official and Provisional Methods of Analysis. *U. S. Dept. Bur. Chem. Bull.* No. 107, 54–55 (1912).

Pervier, N. C., and Gortner, R. A. The estimation of pentoses and pentosans. *Ind. Eng. Chem.*, 15, 1167–1255 (1923). This paper contains a review of the literature and an extensive bibliography of papers on the subject.

XVII. Starches

Expt. 152. Starch from Potato Tubers.—Wash and peel about 600 gm. of potato tubers (*Solanum tuberosum*), macerate them on a fine grater, and thoroughly mix the pulp with an excess of water; filter through muslin, actively manipulating the starch-bearing pulp meanwhile. Allow the filtrate to stand so that the starch may settle. Decant the supernatant liquid and wash several times with distilled water to remove the soluble impurities and any remaining fiber. Dry the starch at room temperature and pass it through a sieve having 100 meshes to the linear inch. Observe some of the dry starch granules under the microscope. What are the successive stages in the complete hydrolysis of starch?

Reichert, E. T. The differentiation and specificity of starches in relation to genera, species, etc. Carnegie Institution of Washington, Publication No. 173, Part I. 1913.

*Small, J. C. Quantitative determination of soluble starch in the presence of starch and its hydrolytic cleavage products. *J. Am. Chem. Soc.*, 41, 107–112 (1919).

Expt. 153. Soluble Starch from Potato Starch.—Soluble starch is the initial product of starch hydrolysis. For a long time Lintner's method has been regarded as the standard for its preparation. By this method the raw starch is treated with 7.5 per cent hydrochloric acid

at room temperature for seven days. As a result, some amylodextrin, erythrodextrin, and copper-reducing substances are also formed; this indicates that the hydrolysis has gone slightly beyond the soluble starch stage. By Small's method, a soluble starch containing a minimum of these substances may be prepared.

Place 20 gm. of potato starch (Expt. 152) in a 300 cc. Erlenmeyer flask and suspend it in 100 cc. of redistilled 95 per cent ethyl alcohol containing 0.75 cc. of concentrated hydrochloric acid. Attach a reflux condenser to the flask, place it in a boiling water bath for 10 minutes and shake constantly to keep the starch in suspension. At the end of this period remove the flask and neutralize the acid alcohol immediately with a N sodium bicarbonate solution so that hydrolysis will stop. The amount of sodium bicarbonate required should be previously determined by titration on a separate sample, using methyl orange as indicator. Filter, wash the starch several times with small quantities of redistilled 95 per cent ethyl alcohol, and dry at room temperature. Why was the potato starch hydrolyzed in ethyl alcohol instead of in water? It has been found that the amount of hydrolysis bears a direct ratio to the hydrogen-ion concentration, which indicates that under proper conditions starch can be completely converted into soluble starch.

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*Small, J. C. A method for the preparation of soluble starch. *J. Am. Chem. Soc.*, 41, 113–120 (1919).

Pringsheim, Hans, und Leibowitz, Jesaia. Zuckerchemie. 332 S. Akademische Verlagsgesellschaft, M. B. H., Leipzig, 1925.

Expt. 154. Coagulation of Soluble Starch.—Soluble starch can be estimated in the presence of unchanged starch, or any or all of its hydrolytic products. The iodine compound of soluble starch may be coagulated by ammonium sulfate. Place 1.5 gm. of soluble starch (Expt. 153) in a 300 cc. Erlenmeyer flask, suspend it in 150 cc. of distilled water and heat to boiling. Everything except the unchanged starch goes into solution. Cool rapidly; filter 110 cc. of the starch solution on a dry filter paper, to remove the insoluble starch; and preserve the unfiltered 40 cc. for Experiment 155. Transfer 100 cc. of the

filtrate to a 300 cc. Erlenmeyer flask, and add 5 cc. of a solution of 4 per cent iodine in 6 per cent potassium iodide, and 100 cc. of a saturated ammonium sulfate solution. Close the flask with a stopper and mix the contents thoroughly. The soluble starch iodine compound coagulates immediately. Filter it through a quantitative filter paper and observe the filtrate.

*Small, J. C. Quantitative determination of soluble starch in the presence of starch and its hydrolytic cleavage products. *J. Am. Chem. Soc.*, 41, 107–112 (1919).

Expt. 155. Determination of the Purity of Soluble Starch.—Using portions of the soluble starch solution (Expt. 154), apply tests for the presence of unchanged starch, of reducing substances, and of erythrodextrin. Perform the tests with Lintner's soluble starch also, and compare results.

(*a*) *Unchanged starch.*—To 10 cc. of the starch solution add a few drops of a solution of 4 per cent iodine in 6 per cent potassium iodide and mix. If unchanged starch is present, the starch iodine compound separates in an almost invisible flocculent form. Filter and observe any precipitate on the filter paper.

(*b*) *Reducing substances.*—Test for the presence of reducing substances, using 6 cc. of Fehling's solution (Expt. 118) and 6 cc. of the starch solution.

(*c*) *Erythrodextrin.*—This may be recognized among the hydrolytic products of starch after the removal of unchanged starch, soluble starch, and amylodextrin. Add to 10 cc. of the starch solution an equal volume of iodine reagent A. Immediately everything in the series above the erythrodextrin separates. Filter off the coagulum; a clear red-brown filtrate results. To this filtrate contained in test tube (1), add dilute sodium thiosulfate solution, drop by drop, until the color just disappears. Then add a sufficient amount of iodine reagent B to supply an excess of iodine. To prepare a control, place in test tube (2) a volume of distilled water equal to that of the decolorized solution, and to this add the same volume of iodine reagent B as was added to tube (1). The erythordextrin red stands out in contrast with the control.

Iodine reagent A.—Dissolve 1 gm. of iodine and 3 gm. of potassium iodide in 500 cc. of saturated ammonium sulfate solution.

Iodine reagent B.—Dissolve 0.3 gm. of iodine and 1.5 gm. of potassium iodide in 100 cc. of distilled water.

*Small, J. C. A method for the preparation of soluble starch. *J. Am. Chem. Soc.*, 41, 113–120 (1919).

Expt. 156. Tests for Starch. Suspend 0.5 gm. of potato starch (Expt. 152) or commercial corn starch in 100 cc. of distilled water and boil for a few minutes. Cool and use portions of this solution for the following tests:

(*a*) *Iodine test.*—To 10 cc. add one or two drops of iodine reagent B (Expt. 155). What is the color of the solution. Heat the solution gently until the color disappears and allow it to cool to room temperature. Note the result.

(*b*) *Ammonium sulfate test.*—To 10 cc. add an equal volume of saturated ammonium sulfate solution and mix thoroughly. Explain the result.

(*c*) *Ethyl alcohol test.*—To 10 cc. add an equal volume of 95 per cent ethyl alcohol and shake. If coagulation does not take place in a short time, add a drop of saturated sodium chloride solution.

(*d*) *Basic lead acetate test.*—To 10 cc. add basic lead acetate solution (Expt. 141), drop by drop with constant shaking, until precipitation is complete. Dextrin is also precipitated by basic lead acetate.

(*e*) *Action of alkali.*—Place 5 gm. of starch in each of two small beakers and suspend each sample in 50 cc. of distilled water. To one beaker add 10 cc. of 12 per cent hydrochloric acid; to the other add slowly, with constant stirring, 10 cc. of 10 per cent sodium hydroxide solution. Explain the result in each case. Is starch a weak acid or a weak base?

(*f*) *Action of formaldehyde.*—Place 1 gm. of starch in a test tube, add 5 cc. of 37–40 per cent formaldehyde solution, mix thoroughly, close with a cork, and allow to stand 2 or 3 days, observing occasionally. At the end of this time, divide the mixture into two portions and suspend each in 5 cc. of distilled water. To one add a few drops of iodine reagent B (Expt. 155) and observe the color occasionally during 24 hours. Allow the other to stand 24 hours; then add a few drops of iodine reagent B, and observe.

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Expt. 157. Quantitative Determination of Starch.—Place 1–4 gm. of sugar-free vacuum-dried residue (Expt. 141) in a 600 cc. beaker. The amount taken depends upon the starch content; in no case should the amount of starch in the sample be more than 0.3 gm. Gelatinize the starch by heating with 200 cc. of distilled water for 40 min. on a steam bath. The mixture should be stirred frequently to prevent the formation of starch aggregates. Cool to 38° C. and add 0.1 gm. of taka-diastase enzyme preparation and 2–3 cc. of toluene. Incubate for 24 hours at 38° C., stirring from time to time to prevent the separation of the starch gel; also replenish the toluene as it evaporates. After digestion is complete, heat the mixture to boiling on a steam bath and boil for 15 min. to inactivate the enzymes. Filter and wash the residue thoroughly by decantation, catching both filtrate and washings in a calibrated 250 cc. volumetric flask. Withdraw aliquots of 30 cc. and determine the reducing sugars (Expt. 139) (Expt. 140).

Thomas found that the equilibrium mixture resulting from the conversion of starch by taka-diastase, contained 2 parts of glucose to one part of maltose. For the cupric reducing methods the amount of starch can be calculated from the total reducing sugars, expressed as glucose, by the formula:

$$g + 0.61m = R,$$

or

$$2m + 0.61m = R,$$

then

$$m = \frac{R}{2.16}$$

and

$$g = \frac{R}{2.16} \times 2.$$

Then starch value for

$$g = \frac{2R}{2.16} \times .900 = S_1.$$

And starch value for

$$m = \frac{R}{2.16} \times .947 = S_2.$$

$S_1 + S_2$ is the total starch content. Where g is the reducing power of glucose, m the reducing power of maltose, R the total reducing power in terms of glucose, 0.900 is the ratio of $\frac{C_6H_{10}O_5}{C_6H_{12}O_6}$, and 0.947 is the ratio $\frac{2C_6H_{10}O_5}{C_{12}H_{22}O_{11}}$.

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XVIII. Inulin

Expt. 158. Inulin from Dahlia Tubers.—Inulin was discovered by Rose in 1805 in the root of elecampane (*Inula helenium*), but the name, inulin, was not given until 1811 when it was suggested by Thomson. It is difficult to obtain in pure form because it is accompanied by the so-called inulides or levulins, which were separated by Tanret into pseudoinulin, synanthrin, helianthenin, and inulenin. These substances form a series with inulin; they differ slightly from each other in solubility and in specific rotation, and they are not convertible one into the other. Dahlia tubers (*Dahlia variabilis*), Jerusalem artichokes (*Helianthus tuberosus*), and chicory root (*Cichorium intybus*) are the best materials for the preparation of inulin.

Extraction.—Grind 2 kg. of clean dahlia tubers as fine as possible in a food chopper. Place 5 liters of distilled water and 60 gm. of precipitated calcium carbonate in a steam-jacketed boiling kettle or other suitable vessel, and add the ground dahlia tubers. The calcium carbonate neutralizes any plant acids and prevents the hydrolysis of inulin. Extract the material at boiling temperature for an hour, adding hot water from time to time to replace that lost by evaporation. Press the liquid through an 8 oz. cotton duck cloth and extract the residue for 30 minutes as before, using 3 liters of distilled water and 15 gm. of calcium carbonate. Again press out the liquid.

Purification.—Add to the combined extracts 20 per cent neutral lead acetate solution until precipitation is complete. Filter over night on large funnels, fitted with fluted filter papers; and then wash with distilled water. Remove the excess of lead with pure hydrogen sulfide,

taking care not to add enough to peptize the precipitated lead sulfide; and then filter.

Crystallization.—Concentrate the solution to about 1000 cc. using the internally heated vacuum apparatus (p. 209); thereafter continue the concentration in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to 16–20 per cent solids, as determined by the Abbé refractometer (Expt. 67). Pour the sirup into a beaker and allow to stand at 0° C. for 2 days or more, until the inulin separates as a crystalline meal. Add to the mixture an equal volume of ice-cold distilled water, stir thoroughly, centrifuge, and discard the supernatant liquid. Wash the crystals once more in ice-cold distilled water; then dissolve them in hot distilled water, and filter while hot. Again concentrate the filtrate under diminished pressure to 16–20 per cent solids and allow crystallization to take place at 0° C. Wash the crystals with an equal volume of ice-cold distilled water, stirring thoroughly, and centrifuge. Then wash the crystals successively with ice-cold distilled water, with 20, 50, 80, and 95 per cent ethyl alcohol, with absolute ethyl alcohol, and finally with dry ether. Dry in the air, and then either in a vacuum desiccator over sulfuric acid for several days or in a vacuum oven at 70° C. for 3 or 4 hours. Determine the ash content. Also determine the specific rotation (Expt. 136) in water at 20° C. For this purpose, dissolve 1 gm. in distilled water in a 50 cc. calibrated volumetric flask; make to volume at 20° C., and polarize in a 200 mm. polariscope tube. The specific rotation should be not less then −35°.

Inulin should be free from reducing sugars. It is hydrolyzed to fructose by boiling with water alone, or with water containing salts, acids, or alkalis, so it is impossible to test for reducing sugars with Fehling's solution. Therefore, the only reliable method is not chemical but bacteriological. When tested with *Bacillus paratyphosus B* or *Bacillus proteus*, according to the method given by Pfanstiehl and Black, it should show no acid or gas production.

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Colin, H. La genèse des lévulosanes chez les végétaux. *Bull. soc. chim. biol.*, 7, 173–180 (1925).

XIX. Mannans

Expt. 159. Quantitative Determination of Mannose and Mannans. —Upon hydrolysis, many of the mannans yield other sugars besides mannose, so that the terms, galacto-mannan, gluco-mannan, fructo-mannan, etc., indicate the sugars obtained. By the addition of phenylhydrazine, the mannose is converted into mannose phenylhydrazone (Expt. 133).

Place in a 300 cc. Erlenmeyer flask 10 gm. of yellow pine (*Pinus ponderosa*) sawdust, which has been prepared by shredding the wood in a disk mill, and then sifting. For sifting, use the standard testing sieves of the American Society of Civil Engineers. The sawdust should pass an 80-mesh, but be retained on a 100-mesh sieve. Add to the flask 150 cc. of hydrochloric acid of sp. gr. 1.025 and hydrolyze by boiling *gently* under a reflux condenser on a sand bath for 3.5 hours. Filter the mixture, collecting the filtrate in a 600 cc. beaker. Rinse the residue into a 200–300 cc. beaker; digest by heating for a few minutes with 100 cc. of distilled water; filter, and collect the filtrate in the beaker containing the original filtrate. Continue this method of extraction until about 500 cc. of filtrate has been obtained. When *cold*, neutralize the filtrate with anhydrous sodium carbonate, using a small portion at a time; slightly acidify the solution with acetic acid, and concentrate on the steam bath to 150 cc. Filter into a 500 cc. glass-stoppered Erlenmeyer flask; wash the residue with a little *cold* distilled water, collecting the washings in the flask. Place this in an ice mixture at a

temperature of about 10° C.; when the contents are perfectly cold, add 20 cc. of distilled water acidified with a few cubic centimeters of glacial acetic acid and 10 cc. of freshly distilled phenylhydrazine (Expt. 12, footnote); shake the mixture vigorously. If the solution is *seeded* with a few crystals of mannose phenylhydrazone, crystallization is facilitated. Allow to stand in the ice mixture 2 or 3 hours, shaking frequently. Filter the mannose phenylhydrazone on a previously prepared and weighed Gooch crucible (Expt. 139), wash several times with small quantities of ice-cold distilled water, and then with acetone to remove resinous impurities. Dry at 100° C., cool in a desiccator, and weigh. The weight of mannose phenylhydrazone, multiplied by the factor 0.666, gives the weight of mannose; or if multiplied by the factor 0.6 gives the weight of mannan. Dissolve the mannose phenylhydrazone in the least possible quantity of hot 75 per cent ethyl alcohol, filter on a small funnel, and allow crystallization to take place. Two or three recrystallizations should result in practically colorless crystals. Determine the melting-point (Expt. 128). This should be 188° C.

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XX. Galactans

Expt. 160. Quantitative Determination of Galactose and Galactans. —Either galactose or the galactans, when heated with nitric acid, are oxidized to mucic acid (Expt. 132). Place in a 150–200 cc. beaker 5 gm. of western larch (*Larix occidentalis*), tamarack or American larch (*Larix laricina*), or yellow pine (*Pinus ponderosa*) sawdust, which has been sifted through an 80-mesh but retained on a 100-mesh standard sieve (Expt. 159). Add 60 cc. of nitric acid of sp. gr. 1.15 and evaporate the mixture to 20 cc. in a water bath at 87° C. Dilute to about 75 cc. with hot distilled water, filter, and wash the residual oxycellulose with hot water until the filtrate comes through practically colorless. This generally occurs when the volume of the filtrate is about 250 cc. Evaporate the combined filtrate and washings to about 10 cc. in a water bath at 87° C. Set aside for several days to allow the mucic acid to separate. Large crystals of an acid, probably oxalic, separate at first;

then a day or two later fine white crystals of mucic acid form. At this point stir the mixture vigorously to facilitate the crystallization of the mucic acid. Twenty-four hours later, dilute with 20 cc. of cold distilled water to dissolve the larger crystals. Allow the mixture to stand another 24 hours, filter the mucic acid on a previously prepared and weighed Gooch crucible (Expt. 139), wash with 50 cc. of cold distilled water, 60 cc. of 95 per cent ethyl alcohol, and several times with ether. Dry for 3 hours at 100° C., cool in a desiccator, and weigh. The weight of mucic acid multiplied by the factor 1.33 gives the weight of galactose; or if multiplied by 1.2 gives the weight of galactan.

*Miyake, K. An improvement of the method for the determination of galactan. *J. Coll. Agr. Tohoku Imp. Univ.*, 4, 337–345 (1911–12).

*Schorger, A. W., and Smith, D. F. The galactan of *Larix occidentalis*. *J. Ind. Eng. Chem.*, 8, 494–499 (1916).

*Dore, W. H. The proximate analysis of coniferous woods. *J. Ind. Eng. Chem.* 12, 476–479 (1920).

Official and tentative methods of analysis of the association of official agricultural chemists. 2nd ed., p. 121. Association of Official Agricultural Chemists, Washington, D. C., 1925.

XXI. Gums and Mucilages

Expt. 161. Mucilage from Flaxseed.—Extract 200 gm. of clean unground flaxseed (*Linum usitatissimum*) with 800 cc. of distilled water for 24 hours. Stir occasionally during the process. Separate the mucilagenous substance from the seeds by filtering through cheesecloth. To the filtrate, add 2 volumes of 95 per cent ethyl alcohol and stir gently. Allow the mixture to stand for an hour. Filter off the coagulated substance on an ordinary funnel; wash with ether; dry at a temperature not above 100° C., and grind to a powder. The yield of mucilage is about 5 per cent of the weight of flaxseed used.

*Neville, A. Linseed mucilage. *J. Agr. Sci.*, 5, 113–128 (1913).

Expt. 162. Isolation of the Hydrolysis Products of Flaxseed Mucilage.—Place the powdered flaxseed mucilage (Expt. 161) in a 500 cc. Erlenmeyer flask and add 250 cc. of 4 per cent sulfuric acid. Boil gently under a reflux condenser for about 6 hours, and then filter off any flocculent matter that remains. Neutralize the filtrate at the boiling temperature with barium carbonate, using an excess, until the solution is neutral to Congo red paper. Filter off the barium sulfate at once on a Büchner funnel and wash with hot distilled water. Then to the filtrate add basic lead acetate solution (Expt. 141), making a series of tests on small portions of the solution

until the required amount of acetate necessary for complete precipitation is determined. Filter, and remove the excess of lead from the filtrate with pure hydrogen sulfide; take care not to add so much that the precipitated lead sulfide is peptized. Again filter, and concentrate the solution in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) to about 25 cc. Pour the sirup into 2 or 3 volumes of 95 per cent ethyl alcohol, stirring meantime; allow to stand over night; then filter. Again concentrate the filtrate under diminished pressure to about 50 cc.; wash it into a 100 cc. calibrated volumetric flask and make to volume. Determine the per cent solids by the Abbé refractometer (Expt. 67). Use portions of this solution for the following tests:

(*a*) Test a portion of the solution for the presence of reducing sugars by using Fehling's solution (Expt. 118).

(*b*) Perform the mucic acid test for galactose (Expt. 131). Filter off the mucic acid which separates, and save the filtrate. Then wash the residue with distilled water; dry, and determine the melting-point of the mucic acid.

(*c*) Heat the acid filtrate obtained from (*b*) over a low flame, and perform the saccharic acid test for glucose (Expt. 132).

(*d*) Test portions of the solution for the presence of pentoses, using the orcinol, phloroglucinol, and aniline hydrochloride tests respectively (Expts. 123, 124, 125).

(*e*) Perform the xylonic acid test for xylose (Expt. 130).

(*f*) With another portion of the solution, prepare arabinose diphenylhydrazone by the method of Neuberg, using a slight excess of diphenylhydrazine; or perform Fischer's test for arabinose by using *p*-bromphenylhydrazine.

*Fischer, E. Ueber einige Osazone und Hydrazone der Zuckergruppe. *Ber.*, 27, 2486–2492. Cf. 2490–2491 (1894).

*Neuberg, C. Ueber die Harnpentose, ein optisch inactives, natürlich vorkommendes Kohlenhydrat. *Ber.*, 33, 2243–2254. Cf. 2253–2254 (1900).

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*Neville, A. Linseed mucilage. *J. Agr. Sci.*, 5, 113–128 (1913).

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Sherrard, E. C., and Blanco, G. W. Some of the products obtained in the hydrolysis of white spruce wood with dilute sulfuric acid under steam pressure. *Ind. Eng. Chem.*, 15, 611–616 (1923).

XXII. Pectic Substances

Expt. 163. Pectin from Grape Fruit Rind.—Pectic substances constitute the middle lamella of all cell walls, and thus serve as the cementing material between the cells. They are most abundant in the parenchymatous tissue of vegetables and fruits, such as turnips and sugar beets, apples and the white inner rind of oranges and grape fruit. Three pectic substances are quite generally recognized: pectose or protopectin, the mother substance; soluble pectin, usually called pectin; and pectic acid. Protopectin is converted into pectin and this into pectic acid by progressive hydrolysis; in nature this is brought about by enzyme action as the fruit ripens, becomes over ripe and decays; in the laboratory, it is produced by the use of dilute acids. The naturally occurring protopectin consists, according to Ehrlich, of the calcium-magnesium salt of a complex anhydro-arabino-galacto-methoxyl-tetragalacturonic acid. The exact combination of the units is uncertain but it is known that, upon hydrolysis, arabinose is easily split off while galactose is not.

Remove the pulp from two grape fruit (*Citrous decumana*) and cut off the yellow outside portion, leaving the thick white inner rind. Grind this to a fine pulp in a food chopper, cover with three or four times its weight of distilled water, allow to soak over night, and then drain, squeezing the pulp in a cheese cloth; this removes most of the bitter glucoside, naringin. Again cover with distilled water and boil for about two hours, adding water when necessary; but boil it down at the end of the period so that it barely covers the material. Drain and squeeze through a cloth. If a small amount of a weak organic acid such as citric is added to the water before boiling, the pectin will be formed more rapidly. Add to the cold filtrate twice its volume of 95 per cent ethyl alcohol; allow the coagulum to settle, and then filter. Dry and pulverize the pectin.

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Sucharipa, R. Protopectin and some other constituents of lemon peel. *J. Am. Chem. Soc.*, 46, 145–156 (1924).

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Expt. 164. Preparation of a Pectin Gel.—Place in a weighed 600 cc. Pyrex beaker 65 gm. of sucrose (granulated sugar), 1 gm. of citric acid, 1.5 gm. of pulverized pectin (Expt. 163), and 100 cc. of distilled water. Heat and stir the mixture in order to dissolve the sugar and pectin; then concentrate the solution by boiling, until it reaches a weight of exactly 100 gm. Pour into a small beaker and allow to stand from 18 to 24 hours. What are the constituents necessary for the formation of a fruit jelly? Does such a gel show syneresis?

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XXIII. Cellulose

Expt. 165. Cellulose Solvents.—(*a*) *Alkaline.*—Place 0.2 gm. of absorbent cotton or of filter paper in a test tube, add 10 cc. of Schweizer's reagent, close the tube with a rubber stopper, and shake until the cellulose is dissolved. Then pour the solution into 50 cc. of dilute hydrochloric acid (1 : 5), stirring the latter meantime. The cellulose separates immediately. How is this method applied commercially?

Schweizer's reagent or cuprammonium solution: Method I.—Dissolve 60 gm. of copper sulfate in 1000 cc. of hot distilled water, add a few drops of dilute sulfuric acid, and allow the solution to cool to 50° C. Add ammonium hydroxide of sp. gr. 0.90 until the precipitation of basic copper sulfate is complete; then neutralize any excess of ammonium hydroxide with a few drops of dilute sulfuric acid. Allow the precipitate of basic copper sulfate to settle, decant the supernatant liquid, and wash the precipitate several times by decantation with hot distilled water, then once with cold distilled water. Place in a suitable container, add 200 cc. of 20 per cent sodium hydroxide solution, and shake thoroughly. Allow the precipitate of cupric hydroxide to settle, decant the supernatant liquid, and wash by decantation with distilled water until the supernatant liquid is free from alkali and sulfate. Filter on a Büchner funnel, finally using the rubber pressure device (Expt. 101), and dry at 45–50° C. The preparation and washing of the cupric hydroxide should be carried out in 3 or 4 hours' time. Place the dry cupric hydroxide in a glass-stoppered bottle; add 800 cc. of ammonium hydroxide of concentration such, that 1000 cc. contains 200–210 gm. of ammonia; and shake thoroughly. On standing, any undissolved material settles to the bottom; this may be removed by filtering through glass wool. For quantitative work, the amount of copper in the solution should be determined, and the theoretical quantity of ammonium hydroxide added, to give a solution containing 11 gm. of copper and 200–210 gm. of ammonia per liter. The composition of the final solution should be checked by analysis.

Method II.—Place 5 gm. of powdered copper [9] in a tall narrow bottle, add 400 cc. of ammonium hydroxide of sp. gr. 0.90. Bubble a current of air through the bottle from 6 to 8 hours, or until the solution will dissolve the specified amount of absorbent cotton.

[9] "Venus," finest copper, manufactured by the United States Bronze Powder Works, may be obtained from hardware stores or from the home office, 222 West 42nd St., New York.

*Schweizer, E. Das Kupferoxyd-Ammoniak, ein Auflösungsmittel für die Pflanzenfaser. *J. prakt. Chem.*, 72, 109–111 (1857).

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(*b*) *Acid.*—Place 0.1 gm. of absorbent cotton in a test tube, add 15 cc. of zinc-chloride-hydrochloric acid solution, and stir the mixture with a glass rod until the cellulose is dissolved. Then pour the transparent gelatinous product into 50 cc. of distilled water, stirring the latter meantime. Allow to stand for half an hour and record the results.

Zinc-chloride-hydrochloric acid solution.—Dissolve zinc chloride in twice its weight of concentrated hydrochloric acid.

*Cross, C. F., and Bevan, E. J. A new solvent for cellulose. *Chem. News*, 63, 66 (1891).

(*c*) *Zinc chloride.*—Place 0.2 gm. of absorbent cotton in a test tube, add 15 cc. of saturated zinc chloride solution, and heat in a boiling water bath until the cellulose is dissolved. Then pour the solution into 100 cc. of distilled water, stirring the latter meantime. Allow to stand for half an hour and record the results.

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Expt. 166. Cellulose Acetate.—Place 20 cc. of glacial acetic acid in a 100 cc. Erlenmeyer flask, add 6 cc. of acetic anhydride, 3 drops of

concentrated sulfuric acid, and 0.5 gm. of absorbent cotton. Press the cotton into the solution with a glass rod until it is thoroughly saturated. Close the flask with a cork and allow it to stand over night or longer. For what purpose was the sulfuric acid used? The acetylation product is soluble in the reaction mixture, but it may be recovered in the solid state by pouring it into water. Pour the clear solution in a thin stream into 500 cc. of distilled water, which is stirred during the process. Filter the cellulose acetate on a Büchner funnel, and allow to dry. Place one-half of the dry product in a test tube, add 20 cc. of chloroform, and stopper. If allowed to stand for some time, the cellulose acetate will go into solution. Pour this solution upon a large watch glass and allow the chloroform to evaporate. Then pour sufficient distilled water into the watch glass to cover the cellulose acetate, and allow to stand for 1 or 2 minutes. Carefully loosening the edge of the film, remove it slowly from the glass; dry, and test its burning qualities. Using portions of the other half of the cellulose acetate, test its solubility in acetone, ethyl alcohol, ether, and glacial acetic acid. Is it possible to obtain pure triacetate when sulfuric acid is used? What are some of the commercial uses of cellulose acetate?

*Miles, G. W. Cellulose derivative and process of making the same. U. S. Patent 838,350. Dated Dec. 11, 1906.

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Hibbert, H. Studies on the chemistry of cellulose. I—The constitution of cellulose. *J. Ind. Eng. Chem.*, 13, 256–260 (1921).

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Expt. 167. Color Tests for Lignin.—(*a*) *Phloroglucinol test.*—Place a few drops of phloroglucinol solution on shavings from several kinds

of both coniferous and deciduous woods. After a few minutes a magenta color is produced. Make the test also on both newspaper and filter paper. Account for the results. Other phenols, such as orcinol, resorcinol, and pyrogallol, which also have two hydroxy groups in the *m*-position to each other, give strong red, violet, and blue colors respectively, but in decreasing order of rapidity of color formation. The colors produced by these phenols are apparently due to the presence of an aldehyde in the wood, and Klason concludes that this is coniferyl aldehyde.

Phloroglucinol solution.—Prepare 2 per cent phloroglucinol in 6 *N* hydrochloric acid.

(*b*) *Mäule's Permanganate Test.*—Wood is successively treated with neutral potassium permanganate, dilute hydrochloric acid, and finally with either ammonium hydroxide or sodium bicarbonate solution. Cover shavings from several kinds of deciduous and coniferous woods with 1 per cent potassium permanganate solution and allow to stand for 15–20 minutes. During this time the permanganate deposits manganese dioxide on the wood. Wash out the potassium permanganate and place the shavings in hydrochloric acid of sp. gr. 1.06. The hydrochloric acid reacts with the manganese dioxide to produce chlorine, and this in turn causes a chlorination of some compound in the wood. After the manganese dioxide has been dissolved, wash the shavings thoroughly with distilled water; then, add a few drops of either ammonium hydroxide or sodium bicarbonate solution. A deep red color develops in the deciduous woods, but only an indefinite brown in the coniferous woods. If gingko (*Ginko biloba*) is excepted, the test furnishes a means of distinguishing between deciduous and coniferous woods.

*Mäule, C. Das Verhalten verholzter Membranen gegen Kaliumpermanganat, eine Holzreaktion neuer Art. *Beitr. wissensch. Botanik*, 4, 166–185 (1901).

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*Crocker, E. C. An experimental study of the significance of "lignin" color reactions. *J. Ind. Eng. Chem.*, 13, 625–627 (1921).

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Expt. 168. Quantitative Determination of Lignin.—Extract 4 gm. of western larch (*Larix occidentalis*) sawdust, which has been sifted through an 80-mesh but retained on a 100-mesh standard sieve (Expt. 159) with ether in an alundum thimble in a Soxhlet extraction apparatus for several hours; and dry at 100° C. Place the extracted sawdust in a 100 cc. Erlenmeyer flask, add ten times its weight of 72 per cent sul-

furic acid, and allow to hydrolyze for 16 hours at room temperature. Transfer the mixture to a 2-liter Erlenmeyer flask, dilute with distilled water to 3 per cent, by weight, of sulfuric acid and boil under a reflux condenser for 2 hours. Filter either in a weighted alundum crucible or in a previously prepared Gooch crucible (Expt. 139), and wash the residue with boiling distilled water until free from sulfates. Dry at 100° C., cool in a desiccator, and weigh. Calculate the percentage of lignin in the sawdust.

*Ost, H., und Wilkening, L. Die Verzuckerung des Zellstoffs. *Chem. Ztg.*, 34, 461–462 (1910).

*Cross, C. F., and Bevan E. J. Researches on cellulose. III. (1905–1910.) pp. 39–41. Longmans, Green and Company, New York, 1912.

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*Mahood, S. A., and Cable, D. E. Comparison of wood cellulose and cotton cellulose. *J. Ind. Eng. Chem.*, 14, 727–731. Cf. 728 (1922).

Ritter, G. J. Chemistry of wood. VII—Relation between methoxyl and lignin in wood. *Ind. Eng. Chem.*, 15, 1264–1266 (1923).

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Powell, W. J., and Whittaker, H. The chemistry of lignin. Part I. Flax lignin and some derivatives. *J. Chem. Soc.*, 125, 357–364 (1924). The authors find that flax lignin contains four methoxyl groups, five hydroxyl groups capable of acetylation, and one aldehyde group.

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INOSITOLS OR CYCLOSES

Expt. 169. Phytic or Inositol Hexaphosphoric Acid.[10]—Phytic acid is one of the most important organic phosphorus compounds found in plants. Upon hydrolysis it yields phosphoric acid and the cyclic hexahydroxy alcohol, inositol, which is isomeric with the hexoses. Anderson has isolated this compound from corn, cottonseed meal, oats, maple seed, and wheat bran; but he has found that the latter is the best source of material for its preparation. The bran should be extracted

[10] Most of the details of this method were obtained from a private communication from Dr. R. J. Anderson.

with 0.5 or 1.0 per cent hydrochloric acid in order to destroy the enzyme phytase, because its hydrolytic action on the phytic acid produces inorganic phosphate. Some of the latter is naturally present in the bran extract, and this must be removed during purification.

Digest 1000 gm. of wheat (*Triticum sativa*) bran in 6 liters of 0.5 per cent hydrochloric acid for about 5 hours with frequent stirring, or allow the mixture to stand over night. Strain through cheese cloth and pass the extract through a Sharples laboratory super-centrifuge, or filter through paper pulp (Expt. 91) on a Büchner funnel. To remove the protein in the extract, add cautiously a 15–20 per cent tannin solution as long as a precipitate forms. Allow this fine voluminous precipitate to settle until it becomes coarse. Pass through the super-centrifuge or filter through paper pulp on a Büchner funnel. The resulting filtrate is practically colorless.

Add to the filtrate a slight excess of the calculated amount of sodium acetate required to react with the free hydrochloric acid present; should a precipitate separate, filter. This gives an acetic acid solution in which to make the subsequent precipitation. Add an excess of a hot concentrated barium chloride solution and allow the precipitate to settle over night; siphon off the supernatant liquid and centrifuge. Finally wash the precipitate thoroughly with distilled water on a Büchner funnel. The filtration proceeds very slowly. Suspend the barium salt in distilled water and decompose it with a *slight* excess of dilute sulfuric acid. Allow the precipitate to stand several hours until it becomes granular; then filter on a Büchner funnel. The filtrate is next precipitated with copper acetate in order to get rid of the excess of sulfuric acid. The copper salt is easily washed free from sulfates with water; it is very slightly soluble in very dilute acids. Add to the clear filtrate a considerable excess of a hot saturated solution of copper acetate, and allow the mixture to stand several hours. Centrifuge; transfer the precipitate to a Büchner funnel, fitted with a hardened filter paper, and wash with distilled water until *free from sulfates,* as indicated by a test with barium chloride. Place the washed copper salt in sufficient distilled water to form a suspension. To accomplish this, grind the salt in a mortar with the water, taking care to break up the larger particles until a uniform mixture is obtained. Decompose this with a rapid current of pure hydrogen sulfide. Filter the mixture on a Büchner funnel and wash with a *little* distilled water. If washed too much, the sulfide will form a colloidal solution and run through the filter. Remove the excess of hydrogen sulfide by passing a current of air through the solution. Test a portion of the free phytic acid solution for inorganic phosphates with ammonium molybdate. If no precipitate separates

after standing one hour at 65° C., the phytic acid may be considered sufficiently pure; the calcium salt may then be prepared.

If further purification of the phytic acid is necessary, remove the inorganic phosphates by repeated precipitation of the substance from dilute hydrochloric acid with alcohol. The inorganic phosphates are more soluble in the dilute acid-alcohol mixture than are the salts of phytic acid. This should be reprecipitated until the resulting product does not give any immediate reaction with ammonium molybdate. Add a barium hydroxide solution to the phytic acid solution until it is distinctly alkaline; filter off the precipitate and wash thoroughly with distilled water. Dissolve the barium salt in the least possible amount of cold 5 per cent hydrochloric acid, filter, and precipitate the phytic acid by adding from 1.5–2 volumes of 95 per cent ethyl alcohol. Allow this to stand over night; filter and wash with dilute ethyl alcohol. Dissolve the precipitate in 5 per cent hydrochloric acid; filter and add gradually to the filtrate a dilute solution of barium hydroxide until a precipitate forms. Again allow to stand over night, filter, wash the residue with distilled water, dissolve it in 5 per cent hydrochloric acid, and reprecipitate with alcohol. Allow to stand over night, filter, wash with dilute alcohol and again dissolve in 5 per cent hydrochloric acid and precipitate with barium hydroxide. These operations may be repeated until a pure, snow-white product is obtained. Suspend the filtered and washed barium salt in distilled water and remove the barium with a *slight* excess of dilute sulfuric acid. Filter off the barium sulfate and precipitate the filtrate with an excess of a hot saturated solution of copper acetate. Filter off the copper precipitate and wash *free from sulfates* with distilled water. Suspend this copper salt in distilled water and decompose it with hydrogen sulfide. Filter off the copper sulfide, wash it with a *little* distilled water and remove the excess of hydrogen sulfide with a current of air. A clear, colorless solution of the free phytic acid is obtained.

To prepare the calcium salt add a concentrated solution of calcium acetate. If the phytic acid solution is sufficiently concentrated, the calcium phytate separates as a white amorphous or granular powder upon the addition of a considerable excess of calcium acetate. In case the calcium phytate does not separate at once, concentrate the solution in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) at 40° C. until a white granular precipitate appears. Filter off the precipitate and wash it with a little *hot* water; then wash thoroughly with 50 per cent ethyl alcohol, and finally with 95 per cent ethyl alcohol and ether. Dry in a vacuum desiccator over sulfuric acid.

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*ANDERSON, R. J. Concerning phytin in wheat bran. *J. Biol. Chem.*, 20, 493–500. Cf. 496–497 (1915).

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CHAPTER VI

GLUCOSIDES

Expt. 170. Detection of Cyanogenetic Glucosides.—It is a characteristic of cyanogenetic glucosides that they yield hydrocyanic acid as one of the products of hydrolysis. When plant tissues containing these glucosides are subjected to the action of anesthetics, such as chloroform or ether, or to the action of frost, hydrolysis takes place, liberating free hydrocyanic acid. The same result occurs when the tissue is subjected to maceration or injury. In each case the glucoside and its corresponding enzyme are brought into contact with each other. Guignard suggested a method for quickly detecting the presence of hydrocyanic acid. Insert a moist sodium picrate paper into a tube containing the plant material and a few drops of chloroform. The sodium picrate paper gradually turns orange, then brick red, if the plant tissue contains cyanogenetic glucosides. The test is very delicate and the rapidity of the change in color depends on the amount of free hydrocyanic acid present. The color, itself, is due to reduction, resulting in the formation of sodium picramate.

(*a*) Place in a test tube a few drops of chloroform and a portion of either a young flax plant (*Linum usitatissimum*) from 3 to 5 inches high, or a young sorghum plant (*Sorghum vulgare*) of the same size. Close the tube with a cork, inserting with it a strip of moist sodium picrate paper. Allow the tube to stand one hour, meantime observing the changes which occur. What are the glucosides contained in flax and sorghum? What are the products of their hydrolysis?

(*b*) Place in a test tube a portion of either a young flax or sorghum plant. Close the tube with a cork, inserting with it a strip of moist sodium picrate paper, and set it in a freezing mixture of ice and salt for about three-quarters of an hour. At the end of this period, remove and allow it to stand at room temperature for one hour.

(*c*) Make a fine powder of one bitter almond (*Prunus amygdalus*, var. *amora*) by scraping it with a knife. Place the meal in a test tube and moisten with distilled water. Close the tube with a cork, inserting with it a strip of moist sodium picrate paper, and allow the tube to stand

one hour. Wild vetch seed (*Vicia angustifolia*) may be used in place of bitter almonds for this experiment. What glucosides are found in bitter almonds and wild vetch? What are their hydrolysis products?

Sodium picrate paper.—Dip strips of filter paper into a 1 per cent picric acid solution and dry. Repeat the operation, using a 10 per cent sodium carbonate solution, and again dry. Preserve these test papers in a stoppered bottle.

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Expt. 171. Amygdalin from Bitter Almonds.—Amygdalin is one of the most important of the cyanogenetic glucosides. It occurs in the seeds of the bitter almond (*Prunus amygdalus*, var. *amara*), and in the seeds of several other species of this genus, but the sweet or cultivated almond (*Prunus amygdalus*, var. *dulcis*) appears to contain almost no amygdalin. Dunstan and Henry have shown that the merest traces of hydrocyanic acid do occur in the seeds and very small amounts in the seedlings. Armstrong and his coworkers found that hydrocyanic acid is present in the earlier stages of the growth of the seeds of the sweet

almond, but disappears as they ripen. The same authors separated amygdalin from the ripe fruit of *Laurus lusitanica* by merely extracting with boiling alcohol.

Pour boiling water over 300 gm. of shelled bitter almonds,[1] allow to stand for about two minutes, then drain, blanch, and remove the testas. Dry the almonds and grind to a fine meal in a food chopper, using the nut butter attachment. To extract the greater part of the oil, transfer the meal to an Erlenmeyer flask, and add 500–600 cc. of ether; allow to stand one hour, shaking frequently. Filter off the ether and extract again with fresh ether. Dry the extracted meal as rapidly as possible where there is no danger from fire. Both amygdalin and the enzyme, emulsin, remain in the meal after the ether extraction. If it is allowed to stand, the emulsin hydrolyzes the amygdalin; therefore, extract the meal quickly. Place it in an Erlenmeyer flask, add double its weight of 95 per cent ethyl alcohol and heat to boiling on the water bath for 10 15 minutes. Filter and extract as before two or three times. This removes the amygdalin. Concentrate the combined alcoholic filtrates in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) until a precipitate begins to form. Cool, add half its volume of ether, and allow the mixture to stand until the amygdalin separates. Filter the crystals on a small Büchner funnel fitted with a hardened filter paper and wash with ether. Dissolve the crystals in a small quantity of hot distilled water and allow crystallization to take place. Amygdalin crystallizes from water in rhombic prisms with three molecules of water of crystallization, and from 80 per cent ethyl alcohol, as shining scales with two molecules of water of crystallization. Auld found that the average specific rotation is $-41.6°$. The yield of pure amygdalin is about 2.5 per cent.

*Robiuet et Boutron-Charlard. Nouvelles experiences sur les amandes ameres, et sur l'huile volatile quelles fournissent. *Ann. chim. phys.*, 44, 352–382. Cf. 379 (1830).

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[1] This material may be obtained from wholesale druggists.

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Expt. 172. Hydrolysis of Amygdalin.—(*a*) *By acid.* Place in a test tube 10 cc. of a 1 per cent amygdalin solution and 2 cc. of 20 per cent hydrochloric acid. Close the tube with a cork, inserting with it a moist sodium picrate paper (Expt. 170), and heat in a boiling water bath for half an hour. Note the odor. Cool the solution and make it just alkaline to litmus paper with a 10 per cent sodium hydroxide solution. Test for reducing sugars by using Fehling's solution (Expt. 118). What are the products of the hydrolysis of amygdalin?

(*b*) *By emulsion.* To 10 cc. of a 1 per cent amygdalin solution, add 0.05 gm. of emulsin (Expt. 203). Close the tube with a cork, inserting with it a moist sodium picrate paper. Incubate the mixture at 37° C. for 6 hours. At the end of this period, note the odor. Test for reducing sugars by using Fehling's solution. Auld found that amygdalin is not completely hydrolyzed by emulsin.

*Caldwell, R. J., and Courtauld, S. L. The hydrolysis of amygdalin by acids. *J. Chem. Soc.*, 91, 666–671 (1907). When dilute hydrochloric acid is used, hydrolysis takes place in two stages.

Armstrong, H. E., Armstrong, E. F., and Horton, E. Studies on enzyme action. XII.—The enzymes of emulsin. *Proc. Roy. Soc.* (*London*) [B], 80, 321–331. Cf. 330 (1908). Note diagram showing the hydrolysis of amygdalin by emulsion at 25° C.

*Auld, S. J. M. The hydrolysis of amygdalin by emulsin. Part I. *J. Chem. Soc.*, 93, 1251–1275 (1908).

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Walker, J. W., and Krieble, V. K. The hydrolysis of amygdalin by acids. Part I. *J. Chem. Soc.*, 95, 1369–1377 (1909). When concentrated hydrochloric acid is used the products of hydrolysis differ from those obtained with the dilute acid.

Expt. 173. A Comparison of the Rate of Hydrolysis of Various Glucosides by Emulsin.—Place in a series of three test tubes, 0.23 gm. of amygdalin, 0.28 gm. of salicin, and 0.27 gm. of arbutin, respectively. Add to each tube 0.1 gm. of emulsin (Expt. 203) and 10 cc. of distilled water); incubate the mixtures at 37° C. for 6 hours. On hydrolysis, each

glucoside produces one molecule of glucose. Now, heat three 2 cc. portions of Fehling's solution (Expt. 118) to boiling in a water bath, and determine the reducing power of each hydrolyzed glucoside by counting the number of drops required to form a precipitate in the Fehling's solution. List the glucosides in the order of their rates of hydrolysis. What are the products of the hydrolysis of each glucoside?

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*Bertrand, G., and Thomas, P. Practical biological chemistry. "Guide pour les manipulations de chimie biologique." Translated by H. A. Colwell. P. 219. G. Bell & Sons, London, 1920.

Macbeth, A. K., and Mackay, J. Studies of the glucosides. Part II. Arbutin. *J. Chem. Soc.*, 123, 717–724 (1923).

Expt. 174. Quercitrin from Lemon Flavin.—This glucoside occurs in the bark of the black oak (*Quercus coccinea*, var. *tinctoria Gray*). Lemon Flavin, a commercial product made from the powdered bark, is the best material for the preparation of quercitrin. Place 20 gm. of Lemon Flavin [2] in a round-bottomed Pyrex flask, add 4 liters of distilled water, attach a reflux condenser and boil for an hour to dissolve the quercitrin. Filter the boiling hot solution on a large Büchner funnel. Add to the extract 20 gm. of the vegetable decolorizing carbon, Norit; heat to boiling for about half an hour and then filter as before. Allow the filtrate to stand from 5 to 7 days until crystallization takes place. Filter off the crystals on a small Büchner funnel, wash with distilled water and dry at 100° C. The crystalline product is pale yellow in color. Determine the melting-point (Expt. 128). This should be 183–185° C. Use a portion of the quercitrin for the following reactions, and preserve the remainder for the preparation of the flavonol pigment, quercetin (Expt. 232).

Reactions of quercitrin.—Prepare a solution by dissolving 0.1 gm. of quercitrin in 100 cc. of hot 95 per cent ethyl alcohol; use a small portion of the solution for each test.

(*a*) Add a small amount of dilute alkali, such as sodium hydroxide. The yellow color becomes more intense. To this yellow solution, add a small quantity of dilute hydrochloric acid. Explain the result.

[2] This may be obtained from J. S. Young & Company, Hanover, Pa.

(*b*) Add a small quantity of dilute ferric chloride solution. A dark brown color is produced.

(*c*) Add a little basic lead acetate solution (Expt. 141). A yellow precipitate of the lead salt is formed.

(*d*) To 5 cc. of the solution add 1 cc. of concentrated hydrochloric acid; then add small quantities of magnesium powder, from time to time. Reduction takes place with the formation of a rose color. Add amyl alcohol and shake. The alcohol extracts the color.

Dyeing properties.—Follow the directions given for the dyeing properties of the flavonol pigment, quercetin (Expt. 232), but use 0.2 gm. of quercitrin in each case for dyeing, instead of 0.1 gm. as recommended for quercetin.

Rigaud, L. Ueber das Quercitrin. *Ann. Chem. Pharm.*, 90, 283–297 (1854). The author prepared quercitrin by extracting black oak bark with 85 per cent alcohol.

Perkin, A. G. The coloring matter of cotton flowers, *Gossypium herbaceum*. Part II. *J. Chem. Soc.*, 95, 2181–2193 (1909).

Viehoever, A., Chernoff, L. H., and Johns, C. O. Chemistry of the cotton plant, with special reference to upland cotton. *J. Agr. Research*, 13, 345–352 (1918). The authors found the glucosides, isoquercitrin and quercimeritrin, present in the cotton plant.

Emerson, R. A. The genetic relations of plant colors in maise. *Cornell Univ. Agr. Expt. Sta., Mem.*, 39, 1921.

Sando, C. E., and Bartlett, H. H. Occurrence of quercetin in Emerson's brown-husked type of maise. *J. Agr. Research*, 22, 1–4 (1921).

*Walton, C. F., Jr. The preparation of rhamnose. *J. Am. Chem. Soc.*, 43, 127–131 (1921).

Sando, C. E., and Bartlett, H. H. Pigments of the Mendelian color types in maise: isoquercitrin from brown-husked maise. *J. Biol. Chem.*, 54, 629–645 (1922).

Expt. 175. Rutin from Poppy Petals.—Extract 100 gm. of air-dried California poppy petals (*Escholtzia californica* Cham.) for several days with ether in a continuous extraction apparatus of the Soxhlet type (Expt. 141). This removes the fats, carotinoids, and other foreign substances. Again extract the petals for several days, using 95 per cent ethyl alcohol to dissolve the glucoside. Evaporate the alcoholic extract to a small volume; pour this into a small quantity of distilled water, and boil to remove the remaining alcohol. Cool and allow to stand. The crude rutin separates as a crystalline precipitate. Filter this on a small Büchner funnel, wash with distilled water, and dry at 100° C. Extract the crystalline mass with ether until the extract is no longer colored; then dissolve it in a large volume of hot distilled water, and allow crystallization to take place. Filter off the crystals, wash with a small quantity of distilled water, dry at 100° C., and determine the melting-point (Expt. 128). This should be 190–192° C. The rutin

crystals are primrose-yellow. The yield of rutin is about 5 per cent of the weight of petals used.

Hydrolysis of rutin.—Upon hydrolysis, rutin yields one molecule each of the flavonol pigment, quercetin, and of the sugars, rhamnose and glucose. Place 2.5 gm. of rutin in a 300 cc. Erlenmeyer flask; add 100 cc. of 5 per cent sulfuric acid, and boil under a reflux condenser on a sand bath, for an hour. After hydrolysis, allow the mixture to stand over night, when the quercetin becomes practically insoluble. Filter on a small Büchner funnel, wash the residue once with cold distilled water, and preserve the filtrate for the identification of the sugars. Remove the residue to a small beaker, mix with sufficient boiling distilled water to form a thick paste, and again filter and wash several times with small quantities of boiling water. Dissolve the quercetin in the smallest possible quantity of boiling 80 per cent ethyl alcohol, and allow crystallization to take place. Filter, wash the crystals with cold distilled water, and dry at 130° C. For methods of identifying the quercetin see Experiment 232.

Identification of Rhamnose and Glucose.—Using precipitated barium carbonate, neutralize the original filtrate to Congo red paper at the boiling temperature. Filter off the barium sulfate, decolorize the filtrate with the vegetable decolorizing carbon, Norit, and again filter. Concentrate the filtrate on the water bath practically to dryness, and prepare the mixed osazones (Expt. 128). The separation of the osazones may be accomplished by taking advantage of their *differential solubility* in acetone. Collect the cold osazones on a small filter and wash several times with small quantities of distilled water. Then wash 5 or 6 times with 3–4 cc. portions of acetone, pouring this filtrate back over the residue to make sure that all the acetone soluble material is in solution. The acetone filtrate contains the rhamnosazone, and the residue, the glucosazone. Evaporate the acetone in the filtrate by placing the test tube in a water bath at about 55° C. Dissolve the crude rhamnosazone in the least possible volume of hot 5 per cent pyridine; filter, keeping the solution at 75–90 ° C., and allow crystallization to take place. Filter off the crystals, dissolve them in the least possible volume of hot 20 per cent ethyl alcohol and again allow crystallization to take place. Filter, dry at 100° C., and determine the melting-point. To obtain pure glucosazone, recrystallize the crude glucosazone first from 5 per cent pyridine and then from 20 per cent alcohol according to the directions given for rhamnosazone. Filter, dry at 100° C., and determine the melting-point.

Weiss, A. Ueber das Rutin, vorgetragen beim Apotheker-Gremium fur Mittelfranken in Baiern. *Pharm. Centr.*, 13, 903–905 (1842). Rutin was isolated first from the leaves of the garden rue, *Ruta graveolens.*

Schunck, E. On a yellow colouring matter obtained from the leaves of the *Polygonum Fagopyrum*, or common buckwheat. *Mem. Lit. Phil. Soc. Manchester* [2], 15, 122–129 (1860). The author identifies the coloring matter as rutin.

Schunck, E. The yellow coloring matter of *Sophora japonica*. *J. Chem. Soc.*, 67, 30–32 (1895). The undeveloped flower buds of *Sophora japonica* yield rutin.

Smith, H. G. On myrticolorin, the yellow dye material of *Eucalyptus* leaves. *J. Chem. Soc.*, 73, 697–701 (1898). The leaves of *Eucalyptus macrorhyncha* contain about 10 per cent of the glucoside, rutin.

Perkin, A. G. The identity of osyritrin, myrticolorin, viola-quercitrin, and rutin. *J. Chem. Soc.*, 97, 1776–1777 (1910).

*Sando, C. E., and Bartlett, H. H. Rutin, the flavone pigment of *Escholtzia californica Cham.* *J. Biol. Chem.*, 41, 495–501 (1920).

Sando, C. E., and Lloyd, J. U. The isolation and identification of rutin from the flowers of elder (*Sambucus canadensis* L.). *J. Biol. Chem.*, 58, 737–745 (1923–24).

Charaux, C. Sur le dédoublement biochimique de la rutine.—Rutinose, nouveau biose, provenant de ce dédoublement. *Bull. soc. chim. biol.*, 6, 631–640 (1924); or Sur le dédoublement biochimique de la rutine. Obtention d'un glucide nouveau, le rutinose. *Compt. rend.*, 178, 1312–1314 (1924). The first reference contains a very complete bibliography of rutin.

Charaux, C. Sur la présence de la rutine dans certains végétaux. Préparation et identification de ce glucoside et de ses produits de dédoublement. *Bull. soc. chim. biol.*, 6, 641–647 (1924).

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Jacobsen, O. Die Glycoside. 174 S. Eduard Trewendt, Breslau, 1887.

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CHAPTER VII

FATS AND ALLIED SUBSTANCES

Expt. 176. Expression of a Vegetable Oil.—To obtain the oil from the fruits or seeds of plants, grind coarsely or crack to liberate the kernels or meats from the hard outer seed coat. The hulls may often be removed by winnowing in a gentle air blast. Grind the cracked seeds until the meal passes a 20-mesh sieve, and cold press in a hydraulic press capable of exerting a pressure of at least 3000 pounds per square inch. Filter the expressed oil on a hot water funnel and preserve in a tightly stoppered bottle away from contact with the air.

Expt. 177. Acid Number.—Rancidity in fats and oils seems to arise from two different changes within the fat. One results from hydrolysis with the formation of free fatty acids; the other, the so-called oxidation rancidity, results from oxidation with the formation of aldehydes, ketones and acids of a lower molecular weight than the acids naturally present. The first type of rancidity is always accompanied by an increase in the titration value of the acidity of the fat and the degree of rancidity can be measured by determining the acid number of the fat or oil. The acid number is defined as *the number of milligrams of potassium hydroxide, required to neutralize the free fatty acids in one gram of a fat, oil or wax.*

Place 10 gm. of a fat or oil (Expt. 176) in a 300 cc. Erlenmeyer flask, taking care that none of the oil or fat gets on the sides of the flask. Add 100 cc. of 95 per cent ethyl alcohol and several glass beads. Connect the flask with a reflux condenser and bring the solution to a boil on a steam or water bath. This insures complete solution of the *free fatty acids.* Do not prolong the boiling as esterification may result. Place the flask in a water bath at 60° C.; allow to cool to that temperature and titrate with 0.10 *N* potassium hydroxide, using 1 cc. of phenolphthalein as an indicator. For high concentrations of fatty acids 0.50 *N* alkali should be used; this prevents unnecessary dilution and cooling of the solution and partial hydrolysis of the resulting soap. Hydrolysis results if the alcohol content falls below 40 per cent and in practice it is advisable to have at least 50 per cent alcohol. The change in color at

the end point is gradual and indefinite. The final color is not permanent because of the neutral esters present and the decolorizing action of the carbonic acid absorbed from the air on shaking. Thorough shaking during the titration is, however, essential. Make duplicate blank determinations on the alcohol and apply this correction to the final results.

One cubic centimeter of 0.10 *N* alkali is equivalent to 5.6108 milligrams of potassium hydroxide or to 0.0282 gram of oleic acid. Report the per cent of free fatty acids calculated as oleic acid.

Pure ethyl alcohol.—See Experiment 111.

Phenolphthalein solution.—Dissolve 1 gm. of phenolphthalein in 35 cc. of pure ethyl alcohol and dilute with 65 cc. of distilled water.

*Holland, E. B., Reed, J. C., and Buckley, J. R. Improved methods for fat analysis. *Mass. Agr. Exp. Sta. Bull.* 166, p. 99, 1915.

*Guthrie, E. S. Concerning rancidity of butter. *J. Dairy Sci.* 1, 218–234 (1917–18).

Expt. 178. Refining of a Vegetable Oil.—Determine the acid number (Expt. 177) for the oil to be refined and calculate the amount of sodium hydroxide required to neutralize the free fatty acids. Add to this an excess of sodium hydroxide varying from 0.1–0.6716 per cent of the weight of the oil, if it contains less than 3 per cent of free fatty acids or add an excess of 0.6716–1.375 per cent of the weight of the oil if it contains up to 20 per cent of free fatty acids.

Place 500 gm. of a representative sample of an oil (Expt. 176) in a 500–600 cc. beaker. If an expressed oil is not available, distill the ether from the extract (Expt. 91 or 92) by heating on a water bath in a distilling flask connected with a condenser, and refine the residual ether-free oil. Heat the oil to 24–27° C. stirring with a mechanical stirrer at about 100 r.p.m. Add the calculated amount of sodium hydroxide while the stirrer is in motion; continue stirring for 15–30 minutes. Apply heat and during an interval of at least 15 minutes, gradually raise the temperature to 42–45° C. Stir continually until a separation of the oil and soap results and then stir for 15 minutes more.

Set the beaker in a water bath at 45° C. for 3 hours and then remove to a cool place so that the soaps may harden. Decant the clear refined oil and filter through a dry filter paper to remove traces of soap. The general procedure must, at times, be modified with different oils. If peanut oil is used, the mixture must be stirred 30 minutes after adding the alkali before heat is applied. If an ether, extracted oil is used in place of an expressed oil, it will usually be highly colored by dissolved plant pigments and will require bleaching or decolorizing. For this

purpose add 10 gm. of fuller's earth, heat to 50–60° C., stir well and filter through a dry filter paper.

*Rules governing transactions in cottonseed and its products, and other seeds and their products as amended and adopted by the Interstate Cotton Seed Crusher's Association at twenty-fourth annual session. p. 98–101. New Orleans, 1920.

Expt. 179. Relative Solubility of Fats in Various Solvents.—Pipet 2 cc. of refined cottonseed or other vegetable oil into each of a series of 10 *dry* test tubes. Test the relative solubility of the fat in the following solvents: ether, petroleum ether, methyl alcohol, 95 per cent ethyl alcohol, absolute ethyl alcohol, chloroform, carbon tetrachloride, carbon disulfide, acetone, and benzene. Shake each test tube and add the solvent slowly from a pipet until the two layers disappear or until a total of 10 cc. has been added. Allow to stand a few minutes and then arrange the solvents in the order of their dissolving power.

Remler, R. F. The solvent properties of acetone. *Ind. Eng. Chem.*, 15, 717–720 (1923).

Expt. 180. Saponification of a Fat.—Place 10 gm. of refined cottonseed or corn oil in a 300 cc. Erlenmeyer flask and add 100 cc. of alcoholic potassium hydroxide solution. To bring about complete saponification, boil the mixture vigorously for an hour under a reflux condenser in a water bath, with occasional rotation. Sodium hydroxide, which has greater basicity than potassium hydroxide, may be used but the resulting hard soap is less soluble. Cool and add to the flask 150 cc. of distilled water. Write the equation for the saponification of a fat. What is the saponification number?

Alcoholic potassium hydroxide solution.—To 1000 cc. of 95 per cent ethyl alcohol add 50 cc. of a saturated potassium hydroxide solution slowly, and with shaking, to prevent an appreciable rise in temperature. Allow to stand at least 24 hours and filter.

(*a*) "*Salting out*" *soap.*—To 10 cc. of the dilute saponified solution add an equal volume of saturated sodium chloride solution. Filter off the coagulum and dissolve it in 100 cc. of distilled water. Filter again if necessary. Explain the mechanism of the "salting out" process. Are there any analogies to such action? To one portion of the solution, add a few drops of a saturated calcium sulfate solution; to another, add magnesium sulfate solution, and to a third, calcium bicarbonate solution. Explain what occurs in each case.

(*b*) *Separation of the fatty acids.*—To the remainder of the dilute saponified solution add dilute sulfuric acid (1 : 4) until it is distinctly acid to litmus paper. Write the equation. Boil the mixture under a reflux condenser in a water bath, with occasional shaking, until two layers

separate and the lower becomes practically clear. Immerse the flask in cold water [1] to solidify the mixed fatty acids; pour off the solution and preserve it for the preparation of glycerol. To remove the excess of sulfuric acid, add 150 cc. of distilled water to the solidified acids in the flask, boil as before, cool, and decant. Test the solubility of the fatty acids in ether, and in 95 per cent ethyl alcohol (Expt. 179).

(*c*) *Emulsification of fat by soap.*—(1) Place a small quantity of the mixed fatty acids in a test tube, dissolve them in a little 10 per cent sodium hydroxide solution, add a few cubic centimeters of distilled water and shake. What happens? Add a few drops of a mineral oil, such as paraffin oil, and shake vigorously. Explain the cleansing or detergent action of soap.

(2) Mix 1 cc. of paraffin oil with 2 cc. of 10 per cent sodium hydroxide solution and shake vigorously. Compare the result with the previous one and explain.

(3) Place in a test tube 10 cc. of distilled water and 4 or 5 drops of 10 per cent sodium hydroxide solution, add 1 cc. of the olive oil-oleic acid mixture (Expt. 53) and shake vigorously. Explain the result.

(*d*) *Separation of glycerol.*—Filter the solution decanted from the mixed fatty acids (*b*), carefully neutralize the filtrate with 10 per cent sodium hydroxide solution, and evaporate to dryness on the water bath. Extract the cold residue with 20 cc. of 95 per cent ethyl alcohol, stirring thoroughly; allow to stand for a few minutes, and filter. Extract the residue a second time and filter. Evaporate the combined alcoholic filtrates on the water bath. The sirup remaining is impure glycerol. Why is alcohol used for the extraction? Purer glycerol results if absolute alcohol is used.

McBain, J. W. Colloid Chemistry of Soap. Part I.—Solutions. Third Report on colloid chemistry and its general and industrial applications, pp. 2–31. *Brit. Assoc. Advancement Sci. Repts.*, 1920.

Fischer, M. H. Soaps and proteins: their colloid chemistry in theory and practice. John Wiley & Sons, Inc., New York, 1921. History and theory of "salting out" of soaps, pp. 110–116; the foaming, emulsifying, and washing properties of soaps, pp. 136–160.

McBain, J. W., and Walls, E. Colloid chemistry of soap. Part II.—The soap boiling processes. Fourth report on colloid chemistry and its general and industrial applications, pp. 244–263. *Brit. Assoc. Advancement Sci. Repts.*, 1922.

Chapin, R. M. Test for comparing detergent efficiencies of soaps. *Ind. Eng. Chem.*, 17, 461–465 (1925).

Chapin, R. M. Fundamental principles of detergent action revealed by the graphite test. *Ind. Eng. Chem.*, 17, 1187–1191 (1925).

[1] A flat-bottomed sink, which has the outlet closed with a one-hole stopper carrying a straight piece of glass tubing to regulate the depth of the water, is a very satisfactory cooling bath.

Expt. 181. Tests for Glycerol.—(*a*) *Color tests.*—When glycerol is oxidized by bromine, it yields a mixture of glyceric aldehyde and dihydroxyacetone. The latter is converted into methylglyoxal by sulfuric acid, which causes dehydration. Methylglyoxal gives very sensitive color reactions, which permit small quantities of glycerol to be identified in fermentation studies and other biological processes.

Place in a test tube 0.1 cc., or 1 cc. of a 10 per cent solution, of pure glycerol, and add 10 cc. of freshly prepared saturated bromine water. Immerse the tube in a boiling water bath for 20 minutes and then, if necessary, boil over a free flame until the bromine fumes have all been driven off. Use the resulting solution for the following tests:

(1) In the presence of potassium bromide, an alcoholic solution of salicylic acid gives a violet red color and shows two absorption bands in the blue and yellow parts of the spectrum. Place in a test tube 0.1 cc. of a 4 per cent potassium bromide solution, 0.4 cc. of the glycerol test solution, 0.1 cc. of a 1 : 20 ethyl alcohol solution of salicylic acid, and 2 cc. of concentrated sulfuric acid. Shake and, if necessary, heat for two minutes in a boiling water bath.

(2) Alcoholic solutions of resorcinol, thymol, and β-naphthol also react with the glycerol test solution. Place in 3 test tubes respectively, 0.1 cc. of ethyl alcohol solutions of 1 : 20 resorcinal, 1 : 20 thymol, and 1 : 50 β-naphthol. Add to each 0.4 cc. of the glycerol test solution and 2 cc. of concentrated sulfuric acid and shake the tubes. It may be necessary to heat the β-naphthol mixture for two minutes in a boiling water bath. The following results will be apparent: resorcinol gives a blood red color, and shows two absorption bands in the blue and yellow parts of the spectrum; thymol gives a wine red color; and β-naphthol an emerald green color with a green fluorescence. There are two absorption bands in the green and red parts of the spectrum. When diluted with glacial acetic or concentrated sulfuric acids, all of these tests produce less intense colors.

Repeat the color tests, using one or two drops of glycerol (Expt. 180*d*). Compare the results with those obtained from pure glycerol.

Denigès, G. Réactions colorées de la dioxy-acétone. *Compt. rend.*, 148, 172–173 (1909).

*Denigès, G. Nouvelles réactions de la dioxy-acétone. *Compt. rend.*, 148, 282–283 (1909).

*Denigès, G. Théorie des réactions colorées de la dioxyacétone en milieu sulfurique. *Compt. rend.*, 148, 422–424 (1909).

*Denigès, G. Nouvelles réactions très sensibles pour la recherche et l'identification de la glycérine. *Compt. rend.*, 148, 570–572 (1909).

(*b*) *Acrolein test.*—Mix in a mortar one or two drops of glycerol (Expt. 180*d*) with a small quantity of powdered potassium bisulfate.

Heat the mixture cautiously in a *dry* test tube and note the peculiar irritating odor. What is the function of the potassium bisulfate? Write the equation. Perform the same test using cottonseed oil. Will this test serve to distinguish fats and oils from mineral oils, oily esters, and other substances having the physical properties of fats and oils?

Witzeman, E. J. The preparation of acrolein. *J. Am. Chem. Soc.*, 36, 1766–1770 (1914). It is prepared from glycerol and anhydrous magnesium sulfate.

Expt. 182. Kreis' Test for Detection of Oxidation.—Place 2 cc. of an oil or melted fat in a test tube, add 2 cc. of a freshly prepared 1 per cent solution of phloroglucinol in ether, and 2 cc. of concentrated hydrochloric acid; close with a rubber stopper, shake vigorously for 30 seconds, allow to stand, and note the appearance of any color. If the fat has been oxidized, a red or pink color will appear in the acid layer. To determine the intensity of the test, use the material in the test tube and dilute it with a non-reacting oil, such as U. S. P. liquid petrolatum; judge the intensity by the degree of the dilution of the original fat, at which a color ceases to be observed. A recognizable red or pink shade is regarded as a test, while a faint orange or yellow is not. The intensity is recorded in terms of the highest dilution at which color is obtained. For example, if the highest dilution, at which color results, is obtained with 1 part of fat and 10 parts of the non-reacting oil, the intensity of the reaction is recorded as 1 to 10. Try two dilutions, one containing 1 part of the unknown fat to 5 parts of the non-reacting oil, and the other, 1 part to 10; if the end-point is not reached, continue the dilution.

*Kreis, H. Über Farbenreaktionen fetter Öle. *Verhandlungen der Naturforschenden Gesellschaft in Basel*, 15, 225–251 (1903–04).

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*Kerr, R. H. Chemical tests for the detection of rancidity. *Ind. Eng. Chem.*, 10, 471–475 (1918).

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Powick, W. C. Compounds developed in rancid fats, with observations on the mechanism of their formation. *J. Agr. Research*, 26, 323–362 (1923).

*Kerr, R. H., and Sorber, D. G. The analytical detection of rancidity. *Ind. Eng. Chem.*, 15, 383–385 (1923).

Holm, G. E., and Greenbank, G. R. Quantitative aspects of the Kreis test. *Ind. Eng. Chem.*, 15, 1051–1053 (1923).

Holm, G. E., and Greenbank, G. R. Quantitative aspects of the Kreis test—II. *Ind. Eng. Chem.*, 16, 518 (1924).

Greenbank, G. R., and Holm, G. E. Some factors concerned in the autoxidation of fats. With special reference to butter fat. *Ind. Eng. Chem.*, 16, 598–601 (1924).

Expt. 183. Halphen's Test for Cottonseed Oil.—Use 3 test tubes, and place in one 3–5 cc. of pure cottonseed oil; in the second, 3–5 cc. of a mixture of a pure oil, such as corn (Mazola) or olive oil, and a little cottonseed oil; and in the third, 3–5 cc. of an oil suspected of containing a trace of cottonseed oil. Add to each an equal volume of the reagent, close with stoppers, and heat in a boiling saturated sodium chloride solution. In the tube containing the pure cottonseed oil, the characteristic red or orange-red color develops in a few moments. A mixture, which contains as small an amount of cottonseed oil as 1 per cent, will give the test, but it may be necessary to heat it from 1 to 2 hours before the color develops. To a certain extent the intensity of color is proportional to the amount of cottonseed oil, so comparative tests with pure oil will give an idea of the quantity present. The chromogenic substance, which gives the color, has the property of passing into the fat of an animal. Lard, lard oil, and butter fat obtained from animals fed on cottonseed meal will give the test; the butter fat of a cow will respond to the test 48 hours after she has begun to feed on the meal. A negative test is not proof of the absence of cottonseed oil. If the oil has been heated to 200–210° C. for some time, the intensity of the reaction is greatly diminished; and if heated to 250° C. for 10 minutes, it will not respond to the test. Hydrogenated cottonseed oil will not react.

Halphen's reagent.—Prepare a 1 per cent solution of sulfur in carbon disulfide. Just before using, mix with it an equal volume of amyl alcohol.

*Halphen, G. Réaction caractéristique de l'huile de coton. *J. pharm. chim.* [6], 6, 390–392 (1897).

*Raikow, P. N., und Tscherwenivanow, N. Untersuchungen über die Farbreactionen von Becchi und Halphen zur Identificirung des Baumwollsamenöles. *Chem. Ztg.*, 23, 1025–1028 (1899).

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Raikow, P. N. Beitrag zur Aufklärung der Halphen'schen Farbreaction zur Identificirung des Cottonöles. *Chem. Ztg.*, 24, 562–563; 583–585 (1900).

Raikow, P. N. Zur Frage nach dem Chemismus der Halphen'schen Reaction auf Cottonöl. *Chem. Ztg.*, 26, 10–11 (1902).

Expt. 184. Iodine Absorption Number.—*The iodine absorption number is the number of centigrams of iodine absorbed by* 1 *gram of a fat*

or oil. This constant is most valuable in identifying and differentiating oils; as well as in indicating the group to which an oil belongs. It is not as easily affected by slight changes in the oil as are some of the other constants.

The unsaturated fatty acids and their glycerides absorb halogens to form addition products, chiefly; this property serves as a basis for their quantitative determination. Iodine is the halogen used in practice. The free iodine is absorbed very slowly by the fat or oil, hence active solutions, which contain an unstable compound of iodine, are used with either chlorine or bromine. Wijs' solution, which is to be preferred, contains iodine monochloride as the active agent.

Weigh accurately a melted and filtered fat or oil into a clean dry 300 cc. glass-stoppered Erlenmeyer flask. The amount of fat to be taken for the determination varies inversely with its iodine number. Use about 0.3 gm. of fat if the iodine number is 200 or over, 0.3 to 0.6 gm. between 200 and 100, and larger amounts of fat for still smaller iodine numbers. Add to the flask 20 cc. of carbon tetrachloride or chloroform and after complete solution, 25 cc. of iodine solution, accurately measured from a buret or pipet. For the attainment of constant results it is necessary to add an excess of iodine equal to the amount absorbed. Moisten the stopper with a 10 per cent solution of potassium iodide to prevent the loss of iodine or chlorine by volatilization, but avoid an amount sufficient to run down inside the flask. Allow the flask to stand in a dark place for half an hour, keeping it at a uniform temperature. At the same time carry out two blank determinations in exactly the same manner as the sample except that no fat is used. If, during the absorption period, the solution becomes nearly decolorized, add more of the iodine solution. At the end of the half hour, carefully remove the stopper, add 20 cc. of 10 per cent potassium iodide solution, pouring it over the stopper, and 100 cc. of distilled water. Titrate the excess of iodine with sodium thiosulfate; add this gradually with constant stirring, until the solution becomes pale yellow. Add about 2 cc. of starch solution and continue titration until the blue color has entirely disappeared. Toward the end of the titration, stopper the flask and shake vigorously so that any iodine, which may be dissolved in the carbon tetrachloride or chloroform, may react with the potassium iodide solution. The solution in the stoppered flask will remain clear for some time, although the blue color will finally develop again because of the splitting off of iodine. Subtract the number of cubic centimeters of sodium thiosulfate solution required in the titration of the sample, from the average number of cubic centimeters required by the two blanks; this gives the thiosulfate equivalent of the iodine absorbed by the weight

of the sample used in the determination. Calculate the number of centigrams of iodine absorbed by 1 gram of the sample; this is equivalent to the per cent of iodine absorbed.

Wijs' iodine solution.—Dissolve 13 gm. of resublimed iodine in 1 liter of chemically pure glacial acetic acid. Titrate a 20 cc. portion with sodium thiosulfate. Reserve about 50 cc. of the solution. Pass into the remainder washed and dried chlorine gas until the iodine has been converted into the monochloride. Wash the chlorine gas by passing it through water and dry it by passing it through concentrated sulfuric acid; the presence of moisture spoils the solution. Sufficient chlorine gas can be generated for the preparation of 4 liters of iodine solution from 44.5 gm. of sodium chloride, 55.5 gm. of manganese dioxide and 150 cc. of sulfuric acid (1 : 1). The reaction is complete when the original thiosulfate titration of the solution is just doubled. Last add 25 cc. of the original solution to react with any free chlorine dissolved in the liquid, and to give a slight excess of iodine.

Sodium thiosulfate solution.—Prepare a 0.1 *N* solution according to the method (Expt. 140).

Potassium iodide solution.—Dissolve 75 gm. of chemically pure potassium iodide in distilled water and make to 500 cc.

Potassium dichromate solution.—Dissolve 3.8633 gm. of dry chemically pure potassium dichromate in distilled water and dilute to 1 liter. The solution should be checked against pure ferrous ammonium sulfate or iron wire. If the dichromate is dry, 1 cc. is equivalent to 0.01 gm. of iodine. The solution will keep almost indefinitely and is used for standardizing the thiosulfate solution.

Starch solution.—Mix 0.5 gm. of soluble starch with cold distilled water to a thin paste; pour this into about 100 cc. of boiling water, boil for several minutes and cool to room temperature.

Standardization of the sodium thiosulfate solution.—Measure accurately 20 cc. of potassium dichromate solution and 10 cc. of potassium iodide solution into a 300 cc. Erlenmeyer flask; add to this 5 cc. of concentrated hydrochloric acid. Dilute this mixture with 100 cc. of distilled water and slowly add sodium thiosulfate solution to the flask until the yellow color almost disappears. Add about 2 cc. of the soluble starch solution or sufficient to produce a marked blue coloration. Continue the titration cautiously until the blue color just disappears, leaving a bright green solution. Calculate the iodine value of 1 cc. of sodium thiosulfate, according to the following equations:

$$K_2Cr_2O_7 + 14HCl + 6KI = 2CrCl_3 + 8KCl + 7H_2O + 3I_2$$

$$3I_2 + 2Na_2S_2O_3 = Na_2S_4O_6 + 2NaI$$

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Lewkowitsch, J. Chemical technology and analysis of oils, fats, and waxes. 5th ed., 1, p. 407. The Macmillan Company, 1913.

Expt. 185. Hydrogenation of an Oil.—The conversion of an oil, which is liquid at the ordinary temperature into a fat which is solid under the same condition, is called hydrogenation. It is done by adding hydrogen to the molecules of the glycerides of the unsaturated acids, such as oleic, linoleic, and linolenic, in the presence of a catalyst; this transforms each into the glyceride of the corresponding saturated acid, stearic. In the commercial lard substitute, hydrogenation is not completed, so the product consists of a mixture of unsaturated glycerides with just enough tristearin to make it congeal at the ordinary temperature. The best catalyzers are nickel, palladium, and platinum; but nickel oxide, such organic salts of nickel as the formate, acetate, and lactate, and metals, such as copper, iron and cobalt, have been used.

Place in a Pyrex test tube, 250 × 25 mm., 4 or 5 gm. of powdered nickel formate and 75 or 100 cc. of either refined cottonseed or corn oil. Close the tube with a 3-hole stopper, carrying a thermometer which is partially immersed in the oil, a glass inlet tube drawn to a point and extending to the bottom of the test tube, and an outlet tube. Connect the inlet tube with a cylinder of compressed hydrogen; heat the mixture in the test tube to 170° C. in an oil bath, and pass a slow stream of hydrogen through it for 2–2.5 hours. During this time gradually raise the temperature from 170 to 200° C. At the end of the reduction period, filter the hot mass, several times, through dry filter paper in a hot-water jacketed funnel to remove the catalytic agent held in suspension. Preserve this for use in a subsequent hydrogenation by suspending it in oil. Permit the filtrate to solidify and then take the melting-point. Also determine the iodine absorption number (Expt. 184) of the oil, both before and after hydrogenation. How does the iodine number of the hydrogenated cottonseed oil compare with that of the commercial lard substitute, Crisco?

The hydrogenation of butter fat may be carried out by the same method. The resulting product is snow white and has neither the taste nor odor of butter. Prepare the butter fat from butter as follows: Place the butter in an oven at 60° C., and allow it to remain several hours until the water and curd have separated from the butter fat. Filter the clear, supernatant fat through a dry filter paper either in a hot-

water jacketed funnel or in an oven at about 60° C. If the filtered fat is not perfectly clear, refilter.

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Expt. 186. Hexabromide Test.—After preliminary hydrolysis of a fat or oil, two groups of fatty acids, the saturated and unsaturated, may be separated by the lead-salt-ether method. The lead salts of the solid saturated acids, such as myristic, palmitic, stearic, arachidic, and lignoceric are practically insoluble in ether, while those of the liquid unsaturated acids, oleic, linoleic, linolenic, and clupanodonic are soluble. Jamieson and Baughman find, however, that this method does not effect a complete separation of the saturated and unsaturated acids, for the saturated acid fraction always absorbs iodine, showing the presence of unsaturated acids. The appropriate corrections should, therefore, be applied. After separating the two groups, the saturated acids may be converted into their methyl esters and subjected to fractional distillation under diminished pressure; while the unsaturated acids may be converted into their bromine addition derivatives which are then separated from each other according to their solubility in various solvents. Oleic dibromide is very soluble in petroleum ether, while linoleic tetrabromide is only slightly soluble. The hexabromide, the bromine derivative of linolenic acid, is insoluble in ether, while both the dibromide and tetrabromide are soluble. These facts suggest that the two latter compounds may be determined in the mother liquor from the ether insoluble hexabromide. The preparation and separation of the three bromine addition derivatives are performed according to the method of Eibner and Muggenthalor; but great care must be exercised for otherwise,

according to Jamieson and Baughman, considerable tetrabromide remains in solution with the dibromide and a low yield of linoleic acid will result. The octabromide is also insoluble in ether and, if present, is included in the calculation with the hexabromide. It is rarely found except in fish, liver, and blubber oils.

The principles of the above method are used to distinguish between drying oils, such as linseed, and semi-drying oils. Not only the free unsaturated fatty acids, but also their glycerides, form bromine addition derivatives. The hexabromide may, therefore, be prepared either from the linseed oil direct or from the linolenic acid resulting from the hydrolysis of the oil. Steele and Washburn prefer the latter method for two reasons: First, the hexabromide prepared from the free acid is more stable, and has a sharper melting-point and better crystalline structure than the corresponding hexabromide derived from the glycerides. Second, it is not known whether the linolenic acid is present quantitatively as a simple triglyceride or as a mixed glyceride. However, in this experiment the hexabromide will be prepared from the glyceride.

Accurately weigh a glass centrifuge tube about 170 × 30 mm. and place in it 1–2 gm. of either raw, refined, or boiled linseed oil; add 25 cc. of absolute ether, close with a stopper, and cool to 2–3° C. in an ice mixture. Add, drop by drop from a small buret, 3–4 cc. of the brominating reagent. This should produce a deep red color, which is an indication of the correct excess of bromine. Place the centrifuge tube in the refrigerator over night at a temperature below 20° C.; this prevents the formation of substituted bromine derivatives. Again cool the tube in an ice mixture and centrifuge for several minutes. Decant the supernatant liquid, add 10 cc. of ice-cold absolute ether to the precipitate and stir thoroughly with a glass rod in order to wash out the excess of bromine. Cool in an ice mixture, centrifuge as before, and decant the ether. Repeat the washing. A pure white precipitate should remain in the tube. Dry to constant weight at 100° C. Calculate the percentage of hexabromide. Will cottonseed oil form the hexabromide?

Brominating reagent.—Dissolve 5 cc. of bromine in 25 cc. of glacial acetic acid.

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Expt. 187. Sitosterol from Corn Oil.—Oils, fats and waxes consist largely of saponifiable matter, and on boiling with a potassium or sodium hydroxide solution, form water soluble compounds. They also contain some *unsaponifiable matter* which, in oils and fats, includes principally the sterols. The phytosterols are characteristic of plants, while cholesterol is characteristic of animals. In 1897 Burián isolated from wheat and rye embryos a substance which he named sitosterol. This has since been proved to be the phytosterol, characteristic of corn oil. Power and Salway have shown that phytosterols may occur as phytosterol glucosides, for which they have proposed the name, phytosterolins. The standard methods for the determination of unsaponifiable matter include: saponification of the fat; dissolving the resulting soap in water; and extraction of the unsaponifiable matter from the aqueous soap solution with an immiscible liquid, such as ether.

Place 75 gm. of refined corn oil (Mazola) in a round-bottomed flask; then, for each gram of oil, add 2 cc. of 20 per cent alcoholic potassium hydroxide solution. Heat the mixture in a boiling water bath under a reflux condenser for about 2 hours, rotating the flask occasionally. After saponification is complete, dissolve the resulting soap in three volumes of distilled water. Cool the solution and shake it with 200 cc. of pure ether in a separatory funnel. Two layers separate; draw off the soap solution, and remove the ether extract. Again extract the former twice with fresh ether. Combine the ether extracts, which contain the unsaponifiable matter; and remove any traces of alkaline soap by washing many times with distilled water containing a little phenol-

phthalein. To avoid emulsions, shake carefully at first, then more vigorously in subsequent washings. When no reaction toward phenolphthalein is shown, the washing is complete. Dry the ether solution by shaking with granular calcium chloride. Decant or filter from the drying agent; place in a distilling flask, and distill off the ether on a water bath until the volume has been reduced to about 100 cc. Transfer this concentrated ether solution to a small beaker, and continue the evaporation until all of the ether is removed and the residue is practically dry. Dissolve this in the least possible quantity of hot absolute ethyl alcohol and allow crystallization to take place in a test tube, closed with a stopper. Recrystallize the sitosterol two or three times from absolute ethyl alcohol, filter and dry at 100° C. Determine the melting-point (Expt. 128). This should be 137.5° C. Use a portion of the sitosterol for acetylation, and preserve the remainder for the color tests for sterols (Expt. 189).

Acetylation.—Place 0.5 gm. of sitosterol in a large *dry* test tube, add 20 cc. of acetic anhydride, connect with a reflux condenser, and boil for 1 hour. Distill off the excess of acetic anhydride under diminished pressure and purify the acetyl derivative by crystallization from hot absolute ethyl alcohol. Dry at 100° C. and determine the melting-point. This should be 127° C.

Alcoholic potassium hydroxide solution.—Prepare a 20 per cent alcoholic potassium hydroxide solution, using either aldehyde-free ethyl alcohol (Expt. 111) or acetone-free methyl alcohol. After boiling, the solution should be practically colorless; if otherwise, the alcohol has not been aldehyde-free.

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Expt. 188. Cholesterol from Brains.—The method includes the removal of water from the brain with plaster of paris, and its subsequent extraction with acetone. The acetone-soluble material consists principally of cholesterol with a small admixture of lecithin and cephalin.

Thoroughly wash free from all bloody matter 900–1350 gm. of hog, sheep, or ox brains; grind in a food chopper and then work into the mass about three times its weight of plaster of paris. Allow to dry over night; then break into small particles about the size of a grain of wheat; transfer to a percolater, and extract several times with acetone at room temperature. Concentrate the combined extracts in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) and allow to stand over night in the refrigerator. Filter off the cholesterol crystals, dissolve them in hot 95 per cent ethyl alcohol, decolorize the solution with the vegetable decolorizing carbon, Norit, and filter on a hot water jacketed funnel. Allow crystallization to take place, filter, and recrystallize from hot absolute ethyl alcohol. Dry at 100° C. and determine the melting-point (Expt. 128). This should be 148° C. Determine its specific rotation (Expt. 136) in chloroform. According to Hesse, for a 2–8 per cent solution at 15° C., this is $-36.61° + 0.249p$, where p equals the per cent concentration.

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Expt. 189. Color Tests for Sterols.—(*a*) *Whitby's modification of Salkowski's test.*—Place 2 cc. of a chloroform solution containing 1–2 mg. of either sitosterol, a phytosterol (Expt. 187), or cholesterol (Expt. 188) in a *dry* test tube; add 2 cc. of sulfuric acid-formaldehyde solution, and shake thoroughly. Two layers separate almost immediately. Observe that the upper chloroform layer is cherry-red in color, while the lower sulfuric acid layer is brownish-red and shows an intense green fluorescence. Pour the chloroform layer into a dry test tube and add 2 or 3 drops of acetic anhydride. The color changes through purple to a bright blue, which persists for about an hour, and then becomes green. This test is more than ten times as sensitive as the original Salkowski test. What is the mechanism of the color tests for sterols?

Sulfuric acid-formaldehyde solution.—Mix concentrated sulfuric acid and 37–40 per cent formaldehyde solution in the proportion of 50 volumes to one.

(*b*) *Whitby's test B.*—Place 2 cc. of a glacial acetic acid solution containing 0.2–0.5 mg. of either cholesterol or a phytosterol in a dry test tube; add 25 drops of the sulfuric acid-formaldehyde solution (*a*), and shake thoroughly. A rose colored fluorescent solution results. However, the rose color, which has a purple tinge, begins to change after about 2 minutes, and at the end of an hour or more becomes deeper purple.

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(*c*) *Liebermann-Birchard test.*—Place 2 cc. of a chloroform solution, containing 1–2 mg. of either cholesterol or a phytosterol, in a dry test

tube; add 2 cc. of acetic anhydride, 0.1 cc. of concentrated sulfuric acid, and shake thoroughly. Note the series of colors; a momentary rose-red changes quickly to purple, then to deep blue, and gradually to dark green.

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Expt. 190. Lecithin from Egg Yolk.[2]—Dehydrated egg yolk is completely extracted with hot acetone, containing 1 per cent of water; this removes most of the oil and much of the lecithin. Acetone extraction as compared with ether extraction results in a smaller yield of lecithin, but the product contains less cephalin and other impurities, which are difficult to remove.

Place 1000 gm. of dehydrated egg yolk in a 5-liter Erlenmeyer flask, add 2 liters of acetone containing 1 per cent of water, and heat under a reflux condenser on a steam bath for half an hour. Decant the extract through 8 oz. cotton duck and then repeat the extraction of the residue four or five times, using 1.5 liters of acetone containing 1 per cent of water for each extraction. Concentrate the combined extracts either in an internally heated vacuum apparatus (p. 209), or in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136), to 850–1000 cc. From this, precipitate the lecithin as the cadmium chloride salt by the addition of a saturated ethyl alcohol solution of cadmium chloride. Place in the refrigerator for several hours; filter on a Büchner funnel, fitted with a hardened filter paper, and drain as dry as possible, finally using the rubber pressure device (Expt. 101). Transfer the crystalline mass to a beaker, mix with sufficient acetone to form a thick paste and again filter. Repeat this operation until the adhering oil is completely removed.

Suspend the moist crude cadmium chloride salt in about 450 cc. of warm ether and add distilled water until a clear solution results. Allow to stand in the refrigerator for several days and then filter the precipitate on a Büchner funnel. Dissolve this while moist in the minimum quantity of warm toluene. If cerebrosides are present, they will remain undissolved and should be removed by centrifuging. Pour the clear toluene solution into four volumes of *cold* ether containing 1 per cent of water. Precipitation begins in a short time. Allow to stand

[2] Many of the details of this method were obtained from a private communication from Dr. Ida P. Rolf.

over night at 0° C. Either filter or centrifuge to remove the cadmium chloride salt, and wash it with acetone to remove the adhering toluene. About 35 gm. of the dry salt should be obtained. To completely free the salt from amino nitrogen, repeat either or both of these purifications.

Conversion of the cadmium chloride salt into free lecithin.—The cadmium chloride salt is dissolved in chloroform and is then converted into free lecithin by means of a methyl alcoholic solution of ammonia. For every 100 gm. of the cadmium chloride salt use 300 cc. of warm chloroform, and into this solution pour slowly, but with rapid stirring, 400 cc. of methyl alcohol containing 20 gm. of ammonia gas. Allow to stand a short time and then filter off the inorganic precipitate. Concentrate the chloroform methyl alcohol solution almost to dryness in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) at not over 35° C. Add to the lecithin residue distilled water, a little at a time, shaking until a thick emulsion, light brown in color, is formed. Pour this, with stirring, into 800–1000 cc. of acetone, cooled to 5° −C.; this precipitates the lecithin. Allow to stand over night at about 5° −C. and then transfer the precipitate to a beaker and wash it with ice-cold *dry* acetone to remove all of the water. Decant the acetone, dry the residue in a vacuum desiccator over sulfuric acid and preserve in a glass tube, sealed to prevent oxidation. A yield of 50–60 gm. of free lecithin should result from 100 gm. of the cadmium salt.

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CHAPTER VIII

ENZYMES

I. Proteases and Amidases

Peptic activity.—There are various methods for estimating the activity of a pepsin solution, all of which depend upon the fact that the ionized protein is the form which is attacked by the enzyme. This was first suggested by Pauli, and later workers have confirmed his hypothesis. Northrop has further shown that the rate of hydrolysis of protein solutions, of varying concentration but the same hydrogen-ion concentration, is directly proportional to the amount of ionized protein present in the solution and not to the total content of protein. Therefore the optimum hydrogen-ion concentration for the activity of pepsin must coincide with the hydrogen-ion concentration at which the protein solution contains the greatest number of protein ions. When a protein of different isoelectric point is used the range of hydrogen-ion concentration, in which the enzyme is active, shifts.

Several authors have found that pepsin is removed from solution by its substrate. Northrop develops this idea further to show that the optimum zone of hydrogen-ion concentration for the combination of enzyme and substrate corresponds to the optimum for digestion. The amount of pepsin which combines with a given quantity of coagulated egg albumin depends entirely on the hydrogen-ion concentration of the solution in which the egg albumin is suspended. The greatest amount of pepsin is combined when the solution has a hydrogen-ion concentration corresponding to a pH 2.5–3.0. When the protein is insoluble the enzyme combines with it only over that range of hydrogen-ion concentration in which the enzyme is active and in which the protein is ionized.

Much earlier Ringer found that the optimum hydrogen-ion concentration for the digestion of proteins by pepsin varies somewhat with the protein hydrolyzed and with the acid used. He accounted for this by assuming that the degree of imbibition of the protein determines the ease with which it is attacked by the enzyme. However, Northrop finds that with a hydrogen-ion concentration corresponding to a pH 1.0–3.5 the rate of pepsin digestion of egg albumin, gelatin, blood

albumin, casein and edestin is the same in solutions of hydrochloric, nitric, sulfuric, oxalic and phosphoric acids.

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Expt. 191. Estimation of Peptic Activity: Mett's Method.—The action of the enzyme is determined by observing the rate of solution of the substrate. The use of Mett's tubes is not ideal but the method is valuable for obtaining comparative data.

Place in a small Erlenmeyer flask 1 cc. of a pepsin solution and 15 cc. of 0.05 *N* hydrochloric acid. Add two Mett tubes, stopper the flask to prevent evaporation, and place it in an incubator kept at 37° C. for 24 hours. By means of a low power microscope and a millimeter scale determine the length of the column of egg-white digested at each end of the tubes. It is advisable to make duplicate determinations and take the average of the eight readings. It is important that the length of protein dissolved at the ends of the tubes shall not be too great; therefore the concentration of the enzyme used should be such that 2 4 mm. is digested. According to Schütz's law the peptic power is expressed as the square of the number of millimeters of egg-white digested. Consequently, a pepsin solution which digests 2 mm. of egg-white contains four times as much pepsin as one that digest only 1 mm. of egg-white. If the microscope reading gives an average of 2.1 mm. of egg-white digested, the pepsin value for the diluted enzyme preparation would be $2.1^2 = 4.41$, and for the undiluted original pepsin solution, $4.41 \times 16 = 70.56$.

Preparation of Mett tubes.—Cut the white of a fresh egg with scissors, filter through cheese cloth and place the filtrate in a vacuum desiccator

for from two to three hours. This will free it from air bubbles and make it homogeneous. Prepare a number of thin-walled glass tubes of 1–2 mm. internal diameter, clean thoroughly, dry and cut them into lengths of about 10 inches. Suck them full of the egg-white and keep them in a horizontal position. Meanwhile place 5–10 liters of water in a large evaporating dish or other suitable container and heat to boiling. Remove the vessel from the flame and stir with a thermometer until the temperature falls to exactly 85° C. Coagulate the egg-white by laying the tubes horizontally on a wire gauze suspended in the vessel of water and leave them in the water until it has cooled. The egg-white coagulated in this manner is more easily digested than if it were hard boiled; also it is free from air bubbles. Seal the ends of the tubes by dipping them in either melted sealing wax or paraffin: thus prepared they will keep a long time. When ready for use cut the tubes into lengths of about 15 mm. each, and introduce them immediately into the digestion mixture or keep them for a very short time under distilled water. Use only those tubes whose ends are squarely broken off. Since the digestibility of different egg-whites varies widely, a new set of tubes must be standardized against those first prepared if comparable results are to be obtained.

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Expt. 192. Estimation of Tryptic Activity.—The most significant determination with regard to the hydrolysis of proteins by enzymes is the increase in the amino or carboxyl groups. The aliphatic amino groups may be estimated by means of the Van Slyke apparatus (Expt. 110), while the carboxyl can be titrated in 85 per cent ethyl alcohol

with standard alcoholic potassium hydroxide, using phenolphthalein as indicator (Expt. 111).

Suspend 1 gm. of casein in 40 cc. of distilled water, add 8 cc. of 0.10 *N* sodium hydroxide solution, and heat the mixture slowly to 40° C. to hasten its solution. Then heat rapidly to 85–90° C. to destroy any traces of proteoclastic enzymes, which may be contained in the casein. To 10 cc. of this cold casein solution add 0.10 gm. of trypsin and a few drops of toluene, and allow the mixture to stand for 24 hours at 30° C. Then add to this 100 cc. of 95 per cent ethyl alcohol and titrate with 0.10 *N* alcoholic potassium hydroxide solution, using phenolphthalein as indicator. Make a blank determination, using 10 cc. of casein solution and 0.10 gm. of trypsin. To this add immediately 100 cc. of ethyl alcohol and titrate as before. Report the activity of the enzyme in terms of the 0.10 *N* alkali used.

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Expt. 193. Erepsin from Cabbage.—The etiolated leaves of the common white cabbage (*Brassica oleracea*) are a good source for this enzyme. Remove from a firm medium-sized head of cabbage the outer green leaves, which contain a small amount of chlorophyll. Grind the remainder of the cabbage to a fine pulp in a meat chopper and press out the juice in a hand press. 300–500 cc. of juice will be obtained. Filter this to clarify it partially; the filtrate will still be turbid. Dialyze the filtrate (Expt. 2) in a collodion bag against running water for 48 hours, adding toluene as a preservative. Dialysis causes the suspended material to flocculate. Filter off this residue and saturate the clear filtrate with ammonium sulfate. Place in a refrigerator over night or longer. Filter the flocculent coagulum on a small Büchner funnel and carefully wash with a saturated ammonium sulfate solution. Wash the coagulum from the filter paper with about 50 cc. of distilled water. This contains a small insoluble residue, so again filter. Pour the clear solution into a collodion bag and tie firmly just above the contents. Dialyze this against running water until it is free

from ammonium sulfate. This requires from 2 to 5 days. Use toluene as a preservative. The solution is clear, slightly yellow, neutral to litmus and has no odor of cabbage. This partially purified erepsin may be used directly for the tests. The solution deteriorates very slowly if kept in the refrigerator covered with a layer of toluene. When using, withdraw the solution with a pipet.

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Expt. 194. Tests for the Activity of Erepsin. (*a*) *Tryptophane from Witte's peptone.*—Place in each of three test tubes 5 cc. of a solution of 5 per cent Witte's peptone in 1 per cent sodium chloride and 5 cc. of the erepsin solution (Expt. 193). Cover with a layer of toluene and stopper loosely. Digest the contents of each tube at 37–40° C. for 24, 48 and 72 hours respectively. Also digest three blanks containing 5 cc. of the enzyme solution and 5 cc. of distilled water for the same lengths of time. Test the solution in each tube for the presence of tryptophane and compare each original solution with the corresponding blank. Using a pipet, transfer to clean test tubes 5 cc. of the solution from beneath the toluene in each test tube. Make the solutions faintly alkaline and carry out the bromine test for tryptophane (Expt. 88).

Erepsin is active towards Witte's peptone over a considerable range of acidity and alkalinity, but is inhibited by a concentration of hydrogen ions corresponding to the acidity of methyl orange.

(*b*) *Tryptophane from casein.*—To 15 cc. of 0.1 *N* sodium hydroxide solution add 2 gm. of powdered casein and shake vigorously for about 30 minutes or allow to stand several hours, shaking occasionally, until most of the latter is dissolved. Dilute to 60 cc. with distilled water and strain the mixture through glass wool to remove the excess of undissolved casein. The solution is faintly alkaline to litmus. Place in each of three test tubes 5 cc. of the sodium caseinate solution and 5 cc. of the erepsin solution. Cover with a layer of toluene and stopper loosely. Digest the contents of the tubes, run blanks, and make comparisons as described in (*a*).

(*c*) *Tyrosine from silk peptone or peptone "Roche."*—Suspend 10 gm. of silk peptone in 40 cc. of distilled water; neutralize it to phenolphthalein with solid sodium carbonate, and, if necessary, filter. To 5 cc. of this silk peptone solution, add 5 cc. of the erepsin solution, cover

with a layer of toluene and digest at 37° C. for about 72 hours. Crystals of tyrosine should have separated by this time, although this acid shows a tendency to form supersaturated solutions. Observe these crystals under the microscope. Tyrosine may also be identified by the tyrosinase test (Expt. 213).

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Expt. 195. Urease from Jack Bean Meal.—Place about 3 gm. of permutit[1] in a 200–300 cc. Erlenmeyer flask and wash it by decantation, once with 2 per cent acetic acid and twice with distilled water. Add 5 gm. of fine jack bean meal [2] and 100 cc. of 30 per cent ethyl alcohol by volume. Shake gently but continuously from 10 to 15 minutes and filter. The filtrate contains practically all of the urease and a very small amount of other materials. The solution will keep a week at room temperature if not exposed to direct sunlight, and from three to five weeks if kept on ice. The use of permutit frees the extract from ammonia and prevents its development on standing.

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[1] Permutit is prepared by The Permutit Company, 440 Fifth Ave., New York City, and may be obtained from the leading American chemical dealers.

[2] Jack bean meal may be obtained from the Arlington Chemical Company, Yonkers, New York.

Expt. 196. Determination of Urea by Urease.—The method depends upon the principle that the enzyme, urease, is able to transform urea into ammonium carbonate. Place 1 cc. of the urease solution (Expt. 195) in test tube, add 1 cc. of an unknown urea solution, provided by the instructor, and a drop of sodium pyrophosphate, which acts as a buffer. This buffer not only accelerates the decomposition of the urea, but also greatly prolongs the acting period of the enzyme. Digest the mixture in a beaker of water kept at a temperature of 40–55° C. for five minutes, or at room temperature for 15 minutes or longer. Transfer the contents of the test tube to a 200 cc. calibrated volumetric flask and dilute to a volume of about 150 cc. with distilled water. Next prepare a standard by adding 1 mg. of nitrogen in the form of ammonium sulfate to another 200 cc. flask; add to this 1 cc. of the urease solution and dilute to about 150 cc. Then add 20 cc. of Nessler's solution to each flask, dilute to volume and mix thoroughly. Make color comparisons with Nessler tubes or with a Duboscq or Kober colorimeter. By the use of these colorimeters the respective depths of color in two solutions can be accurately measured, and also calculations of the comparative amounts of substances, which quantitatively form these colored compounds, may be made. The depth of the colored solutions through which the light passes is regulated by raising or lowering the cups, the vernier scale at the back of the instrument accurately indicates this in millimeters. Place the standard solution at any convenient depth and match with it the color intensity of the unknown solution by raising or lowering the cups; take the readings on the vernier scale. The amounts of the colored substance in solution are inversely proportional to the depths of the columns of liquid. This is not exactly accurate but is practicable for all ordinary purposes. Thus, if the standard is set at 20 mm. and the solution being examined has the same color intensity at 40 mm. the latter has just one-half the concentration of the standard. The error in reading may be easily reduced to a minimum by using a standard which very nearly matches the unknown solution.

Sodium phosphate solution.—Dissolve 35 gm. of sodium pyrophosphate and 5 gm. of metaphosphoric acid in distilled water and dilute to 250 cc.

Mercuric potassium iodide solution.—Place 150 gm. of potassium iodide and 110 gm. of iodine in a 500 cc. Erlenmeyer flask; add 100 cc. of distilled water and an excess of metallic mercury, 140–150 gm. Shake the flask continuously and vigorously from 7 to 15 minutes or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the red iodine solution becomes visibly pale, though

still red, cool in running water and continue shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This operation takes about 15 minutes. Next decant the solution from the excess of mercury, into a 2 liter calibrated volumetric flask, and wash by decantation with large quantities of distilled water. Finally dilute to volume. If the cooling is begun in time, the resulting reagent is clear enough for immediate use in the preparation of the Nessler solution.

Nessler's solution.—To prepare 3500 cc. of a 10 per cent sodium hydroxide solution, use a completely saturated solution of the substance, which contains about 55 gm. of sodium hydroxide per 100 cc. Decant the clear supernatant liquid and dilute to a concentration of 10 per cent. Titrate a portion to determine that a 10 per cent solution has been obtained with an error of not over 5 per cent. Place the liquid in a large bottle, add 750 cc. of the mercuric potassium iodide solution and 750 cc. of distilled water and mix thoroughly.

The solution contains enough alkali not only to neutralize the acid but also to give a suitable degree of alkalinity for micro Kjeldahl determinations by direct Nesslerization.

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II. Carbohydrases

Expt. 197. Invertase from Baker's Yeast.—Various investigators have shown that liquefaction or autolysis of yeast takes place in the presence of a number of neutral organic liquids. Herzog and Hörth subjected yeast to vapors of acetone, ethyl alcohol, chloroform, ether, toluene and other liquids; they observed that liquefaction occurred in the order in which these substances are named. The rapidity of action depended upon the permeability of the cell membrane to the liquids. Although all these substances cause liquefaction of yeast, others found

later that toluene liberates more invertase than any other liquid. By this method of autolysis of fresh yeast an extract, which has strong inverting power, is obtained.

Break up 5 pounds of starch-free compressed baker's yeast [3] (*Saccharomyces cerevisiae*) by crushing it in the hands. Add 150 cc. of toluene, mix thoroughly and place it in a wide-mouthed glass-stoppered cylinder or bottle, and allow autolysis to take place from 6 to 7 days or longer at room temperature. Filter the autolyzed yeast through filter paper on a large funnel and collect the filtrate in an Erlenmeyer flask containing 25 cc. of toluene. Add sufficient 15 per cent neutral lead acetate solution to the filtrate to insure complete precipitation; centrifuge the solution and decant. Remove the very slight excess of neutral lead acetate by the addition of 2 *N* potassium oxalate, centrifuge and decant. Dialyze the solution immediately in a collodion bag against running tap water for 2 or 3 days, first adding a layer of toluene as a preservative. Tie the bag together just above the contents and suspend it from a glass rod, completely immersing it in water. Finally centrifuge and decant. The dialyzed solution should be colorless, perfectly clear, and neutral to litmus. Place the solution in a sterile ground glass-stoppered bottle, cover with a layer of toluene and keep in the refrigerator. The solution retains its maximum activity for nearly a year. Remove the enzyme solution with a pipet when needed.

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Expt. 198. Rate of Hydrolysis of Sucrose by Invertase.—When measuring the rate of inversion of sucrose by acids, the solution undergoing inversion should be kept in the observation tube in the saccharimeter and read at regular intervals. If the same experiment is carried out for invertase, the saccharimeter reading gives the *apparent* rate of inversion only and not the real rate. In order to obtain the latter it is necessary to take into account the mutarotation of the invert sugar. This correction is negligible in the case of inversion by acids because they accelerate the mutarotation. Hudson has called attention to the fact that the addition of dilute sodium carbonate stops the action of invertase instantly and also accelerates mutarotation so that the rotation can be read at any time after 7 minutes but not later than 2 hours.

Use a sucrose solution having a concentration of 10 gm. per 100 cc. and a hydrogen-ion concentration of pH 4.4–4.5. This is obtained by the use of a buffer mixture of 0.1 *N* acetic acid and 0.1 *N* sodium acetate solutions in the proportions given by Michaelis (Expt. 72). Throughout the experiment keep all solutions at a constant temperature of 25° C.

Initial polarization.—Determine the initial rotation of the sucrose solution by adding the sodium carbonate before the invertase solution, thus rendering the invertase inactive. Take 10 gm. of sucrose, 10 cc. of the buffer solution, 20 cc. of 0.1 *M* sodium carbonate solution and dilute to 100 cc. with distilled water. Then add 1 cc. of invertase solution and mix thoroughly. Withdraw 25 cc. of this mixture, add it to 5 cc. of distilled water and determine the rotation.

Polarization after inversion.—Add to 500 cc. of the sucrose solution 5 cc. of invertase solution and mix thoroughly. It is customary under such conditions to take the time of mixing and of sampling as the mean time of delivery of the pipet used. At the end of each 5, 10, 22, 30, 60, 90, 120, 180 and 300 minute interval and at any time from 2 to 4 days, withdraw a 25 cc. sample of the mixture, stop the reaction and hasten the mutarotation by adding it to 5 cc. of 0.1 *M* sodium carbonate solution. Using a Schmidt and Haensch saccharimeter and a 200 mm. polariscope tube, determine the rotation of each sample after at least 10 minutes and before it has stood 2 hours at 25° C. To determine the

change of rotation in degrees after each interval of time, subtract each reading from the initial rotation. Calculate the percentage of sucrose inverted in each case, using the difference between the final reading and the initial rotation as the total change in rotation. Plot time as abscissae and percentage of sucrose inverted as ordinates. Experimenters have generally believed that the enzyme, invertase, conforms to the unimolecular law, but more recent studies have shown that the rate of hydrolysis of sucrose by invertase is not proportional to the concentration of the substrate, and hence it does not obey this law. Nelson and Hitchcock have developed an empirical equation which expresses the relationship between the percentage and the time of inversion. The experimental results agree very closely with this equation.

Stock buffer solution.—Mix 10 cc. of a normal sodium acetate solution and 16.8 cc. of a normal acetic acid solution and dilute to 100 cc. with distilled water.

Sucrose solution.—Mix 50 gm. of sucrose and 50 cc. of the buffer solution and dilute to 500 cc. with distilled water.

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*Nelson, J. M., and Hitchcock, D. I. Uniformity in invertase action. *J. Am. Chem. Soc.*, 43, 2632–2655 (1921).

Expt. 199. Determination of Sucrose by Invertase.—Dissolve the normal weight, 26 gm., of the unknown substance in distilled water at 20° C., using a 100 cc. calibrated volumetric flask. If an unknown substance is not available dissolve 10 gm. of sucrose in distilled water and dilute to 100 cc. If lead acetate has been used as a clarifying agent, remove the excess of lead from the filtrate with anhydrous sodium carbonate or potassium oxalate and filter.

Direct polarization.—Polarize the solution in a 200 mm. polariscope tube at 20° C. using a Schmidt and Haensch saccharimeter.

Polarization after inversion with invertase.—To 50 cc. of the solution contained in a 100 cc. calibrated flask add acetic acid, drop by drop, until the solution is acid to litmus. Then while rotating the flask, add 10 cc. of invertase solution; nearly fill the flask with distilled water and allow the mixture to stand at a temperature of about 40° C. over

night. Cool the contents of the flask to 20° C. and dilute to 100 cc. with distilled water at the same temperature. Polarize the solution in a 200 mm. polariscope tube at 20° C. Allow the solution to remain in the tube for an hour and repeat the observation. When no change from the previous reading is observed the inversion is complete. Make a blank determination, using distilled water in place of the sucrose solution and taking all readings as before. To determine the correct invert reading, subtract the blank from the observed reading and multiply the difference by two. Calculate the percentage of sucrose by the following formula:

$$S = \frac{100\,(P - I)}{142 - \frac{t}{2} - 0.0065\left[142 - \frac{t}{2} - (P - I)\right]}$$

S = per cent sucrose
P = direct reading
I = invert reading
t = temperature at which invert reading is made

Browne, C. A. Report on sugar. *J. Assoc. Official Agr. Chem.*, 2, 134–143. Cf. 142–143 (1916).

Expt. 200. Determination of Diastatic Power. Lintner's Method. —The method devised by Lintner for the determination of the diastatic power of malt has been used for the determination of the diastatic power of various other materials. Although for accurate work the method has little to recommend it, it is often used for the rapid approximation of the saccharifying value.

Prepare an extract of malt flour by treating 25 gm. of the flour with 500 cc. of water (1 : 20) for 1 hour. Place in each of 10 test tubes 10 cc. of a 2 per cent soluble starch solution (Expt. 140). To the respective tubes add increasing amounts of the diastase solution, 0.1 cc., 0.2 cc., 0.3 cc., etc., shake thoroughly and maintain them at 25° C. for 1 hour. At the end of this time, add 5 cc. of Fehling's solution to each test tube and heat them all in a boiling water bath for 10 minutes. The amount of diastase present is evidenced by the absence of a blue copper color in all tubes to which a certain amount of diastase has been added and the presence of the color in all tubes containing less than this amount. Lintner called the diastatic value of the malt 100 when 0.1 cc. of a malt extract (1 : 20) under the conditions given above produced enough sugar to reduce 5 cc. of Fehling's solution.

Lintner, C. J. Zur Bestimmung der Diastasewirkung. *Z. ges. Brauwesen.*, 8, 281–285 (1885); *Biedermann's Zentr.*, 14, 855–858 (1885).

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*Allen's Commercial Organic Analysis. 4th ed., Vol. I., pp. 137–138. P. Blakiston's Son & Company, Philadelphia, 1909.

Sherman, H. C., Kendall, E. C., and Clark, E. D. Studies on amylases. I. An examination of methods for the determination of diastatic power. *J. Am. Chem. Soc.*, 32, 1073–1086 (1910).

Sherman, H. C., and Schlesinger, M. D. Studies on amylases VI. A comparison of amyloclastic and saccharogenic powers. *J. Am. Chem. Soc.*, 35, 1784–1790 (1913).

Falk, K. G., McGuire, Grace, and Blount, Eugenia. Studies on enzyme action. XVII. The oxidase, peroxidase, catalase, and amylase of fresh and dehydrated vegetables. *J. Biol. Chem.*, 38, 229–244 (1919).

Sherman, H. C., Thomas, A. W., and Baldwin, M. E. Influence of hydrogen-ion concentration upon enzymic activity of three typical amylases. *J. Am. Chem. Soc.*, 41, 231–235 (1919).

Gore, H. C. A polarimetric method for the estimation of diastatic power. *J. Assoc. Official Agr. Chem.*, 7, 364–367 (1924).

Gore, H. C. The effect of hydrogen-ion concentration on the estimation of diastatic power by the polarimetric method. *J. Am. Chem. Soc.*, 47, 281–283 (1925).

Cook, D. H. Temperature coefficients of enzymic activity and the heat destruction of pancreatic and malt amylases. *J. Biol. Chem.*, 65, 135–146 (1925).

Naylor, N. M., Spencer, Mable, and House, Margaret. The preparation and properties of amylase from germinated wheat and rye. *J. Am. Chem. Soc.*, 47, 3037–3039 (1925).

Expt. 201. Determination of Diastatic Power of Wheat Flour: Rumsey's Method.—In this experiment, the term diastase is restricted to mean that enzyme or group of enzymes which produce maltose from the available starch. The neutral or slightly alkaline flour suspension is clarified by the addition of a protein precipitant, such as tungstic acid. Perform the experiment in one laboratory period. Place 10 gm. of wheat flour in each of three 250–300 cc. Erlenmeyer flasks; of these, two are used for the determinations and the third for the blank. Immerse these flasks and a fourth flask, containing about 325 cc. of distilled water, in a water thermostat at 27° C. until the contents of all reach exactly this temperature. Then pipet 100 cc. of the distilled water into two of the flasks containing flour, rapidly rotating them to obtain a thorough suspension. Allow the last few cubic centimeters in the pipet to rinse the flour from the sides of the flask. Quickly replace the flasks in the thermostat, stopper loosely, and allow to remain exactly one hour. Every 10 or 15 minutes during digestion, *gently rotate* the flasks to keep the flour particles in suspension and the temperature equalized. At the end of the hour quickly transfer the contents of the flasks containing the flour suspension to 200 cc. volumetric flasks with distilled water and dilute each to about 175 cc. Although 200 cc. flasks are suggested, no error is introduced by the use of 250 or 300 cc. flasks if the correct factor is applied in the calculation.

When clarifying the solution, add as indicator 5 drops of 0.04 per cent thymol blue (Expt. 72); the solution will be cream-yellow if neutral, or blue if alkaline. Add 3 cc. of a 15 per cent sodium tungstate solution and mix thoroughly with the flour suspension. While constantly shaking the mixture, add drop by drop, from a 1 cc. graduated pipet, sufficient concentrated sulfuric acid to turn the indicator a decided pink, and 2 or 3 drops in excess; if the original flour suspension is nearly neutral, 0.4 cc. of acid is usually sufficient. Enzyme action and the consequent change in sugar content are inhibited by this treatment. Dilute the mixture to the mark, shake thoroughly, and centrifuge until all of the suspended matter has been thrown down. Pipet 50 cc. of the clear supernatant liquid into a 400 cc. Pyrex beaker, and determine the reducing sugars by the gravimetric method of Munson and Walker (Expt. 139). The reduction should be carried out within an hour or two after centrifuging.

To correct for the natural reducing sugars in the flour, make a *blank* determination, using the third flask containing 10 gm. of flour and adding 100 cc. of distilled water at 27° C. from the fourth flask. *Immediately* inhibit diastatic activity by the addition of sodium tungstate and sulfuric acid according to the method described in the preceding paragraph, and then centrifuge.

Subtract the weight of cuprous oxide in the blank from that in the original sample. By reference to the Munson and Walker table, determine from the weight of cuprous oxide, the weight of anhydrous maltose formed. This result corresponds to the diastatic power of 2.5 gm. of flour; to estimate the diastatic power of 10 gm., multiply it by four.

Folin, O., and Wu, H. A system of blood analysis. *J. Biol. Chem.*, 38, 81–110. Cf. 83 (1919). Tungstic acid is used for the precipitation of blood proteins.

*Rumsey, L. A. The diastatic enzymes of wheat flour and their relation to flour strength. *Am. Inst. Baking., Bull.* 8. pp. 48–50, 1922.

Rumsey, L. A. The precipitation of proteins from cereal extracts by sodium tungstate. *Ind. Eng. Chem.*, 15, 270–272 (1923).

Merrill, A. T. A note on the relation of pH to tungstic acid precipitation of protein. *J. Biol. Chem.*, 60, 257–259 (1924).

Expt. 202. Pectinase from the Fungus, Rhizopus.—Investigations have shown that several species of Rhizopus, such as *Rhizopus tritici* Saito and *Rhizopus nigricans* Ehrb. are parasitic on sweet potatoes. These fungi produce a powerful intracellular and extracellular pectinase which dissolves the middle lamella of the sweet potato so that the cells lose their coherence, thereby transforming the potatoes into a soft, watery mass. The cells, however, are not penetrated at least in the

early stages of decay. To produce this enzyme it has been found desirable to use as a liquid culture medium, a decoction made from sweet potatoes. Thus it is possible for the enzyme which diffuses out of the hyphae to become dispersed throughout the medium. When the mycelium is removed, the substrate is ready for the macerating experiments. The mycelium is also utilized for the enzyme which it contains.

To prepare the decoction, peel and slice several sweet potatoes (*Ipomaea batatas*) and add twice their weight of distilled water. Boil for one hour, and then press out the liquid by hand through a cloth; boil a second time and again press out the liquid. Filter the combined liquids by suction through absorbent cotton. Place 250 cc. of the filtrate in a 2-liter Erlenmeyer flask, plug with cotton, and heat in an autoclave at 15 pounds pressure for 20 minutes. The resulting decoction contains some starch and some sugars, but is practically free from cellular structures.

Inoculate the solution in the flask with spores or bits of hyphae from a stock culture of either *Rhizopus tritici* or *Rhizopus nigricans*, which should be in a vigorous state of growth. Incubate in the dark at 37.5° C. for three days. At the end of the incubation period remove the mycelial growth from the flask and filter the substrate through absorbent cotton to remove the remaining threads of hyphae. Wash the mycelial growth in running tap water for 15 minutes; then squeeze out the water by hand. Dehydrate with acetone and ether according to Dox's modification of Albert and Buchner's method for preparing "Acetondauerhefe." Immerse the mycelium for 10 minutes in a large excess of acetone and stir constantly, at the same time tearing the hyphae apart with forceps. Squeeze the material until dry and immerse it in a fresh supply of acetone for two minutes while stirring constantly. Again squeeze it to dryness and stir for three minutes in ether, after which allow it to dry in the air until the odor of ether can not be detected. Preserve the mycelial mass at 9° C. until required for use. Perform two sets of experiments with (*a*) the water suspension of the mycelium and (*b*) the solution on which the fungus grew.

(*a*) Grind 0.50 gm. of the mycelium to a powder in fine quartz sand. Wash the sand in distilled water and heat it in a crucible for two hours before grinding. Transfer the powdered hyphae and sand to a 150 cc. Erlenmeyer flask and add 25 cc. of distilled water. Maintain this suspension for one hour at 37.5° C. in order to bring it to the temperature at which the maceration is to take place. Drop into the flask five raw sweet potato disks, 1 mm. thick and 1.5 cm. in diameter. Always cut the disks from that portion of the potato lying within the fibrovascular

ring, since the tissue here is more uniform. Add about 2.5 cc. of toluene as an antiseptic. Incubate the contents of the flask at 37.5° C. for 5 hours, and note the progress of maceration from time to time. Maceration is considered complete when the disks offer no resistance to pull. Observe the completely macerated tissue under the microscope. In general, it was found that the middle lamellae of sweet potatoes, which have been held in storage for several months, dissolved in about one-half the time required to macerate the tissue of freshly dug sweet potatoes.

(*b*) Use from 25 to 100 cc. of the solution on which the fungus grew, heat to 37.5° C. for one hour, and add five raw sweet potato disks prepared as before. Add toluene and incubate for 5 hours at 37.5° C. Observe and make a record of the progress of maceration. Compare the actions of the intracellular and extracellular pectinase.

Albert, R., Buchner, E., und Rapp, R. Herstellung von Dauerhefe mittels Aceton. *Ber.*, 35, 2376–2382 (1902).

Dox, A. W. The intracellular enzymes of Penicillium and Aspergillus, with special reference to those of Penicillium camemberti. *U. S. Dept. Agr. Bur. Anim. Ind. Bull.*, 120. cf. 38. 1910.

Willaman, J. J. Pectin relations of *Sclerotinia cinerea*. *Bot. Gaz.*, 70, 221–229 (1920).

*Harter, L. L., and Weimar, J. L. Studies in the physiology of parasitism with special reference to the secretion of pectinase by *Rhizopus tritici*. *J. Agr. Research.*, 21, 609–625 (1921).

*Harter, L. L., and Weimar, J. L. A comparison of the pectinase produced by different species of Rhizopus. *J. Agr. Research*, 22, 371–377 (1921).

Weimer, J. L., and Harter, L. L. Influence of temperature on the pectinase production of different species of Rhizopus. *Am. J. Bot.*, 10, 127–132 (1923).

Davidson, F. R. The pectic enzymes. Thesis for Ph.D. Degree, University of Minnesota, June, 1926.

III. Glucosidases

Expt. 203. Emulsin from Sweet Almonds: a β-Glucosidase.— Pour boiling water over 450 gm. of sweet almonds (*Prunus amygdalus*, var. *dulcis*), allow to stand for about two minutes, then drain, blanch, and remove the testas. Dry the almonds and grind to a fine meal in a food chopper using the nut butter attachment. To extract the greater part of the oil, transfer the meal to an Erlenmeyer flask, and add 500–600 cc. of ether; allow to stand one hour, shaking frequently. Filter off the ether and extract again with fresh ether. Dry the extracted meal where there is no danger from fire. To obtain the emulsin extract the meal with 800 cc. of distilled water saturated with chloroform; allow the mixture to stand 24–48 hours, shaking occasionally;

then press it through 8 oz. cotton duck. Precipitate the protein contained in the milky liquid by adding 10 per cent acetic acid until no more precipitate forms, but avoid an excess; then filter. Add to the translucent filtrate 2 volumes of 95 per cent ethyl alcohol and stir. The emulsin separates as a white flocculent coagulum. Allow this to settle for an hour or more; then decant the greater portion of the supernatant liquid; finally, centrifuge and wash with alcohol several times. Dry in a vacuum desiccator over concentrated sulfuric acid. Pulverize the horny mass and preserve it for use in an enzyme synthesis (Expt. 204).

Wöhler, F., und Liebig, J. Ueber die Bildung des Bittermandelöls. *Ann. Pharm.*, 22, 1–24. Cf. 4 and 18 (1837); or Sur la formation de l'huile d'amandes amères. *Ann. chim. phys.*, 64, 185–209. Cf. 188 and 202–203 (1837). The authors discovered and named the enzyme, emulsin.

*Bourquelot, E. Sur l'emploi des enzymes comme réactifs dans les recherches de laboratorie. II. Enzymes hydratants (Hydratases). *J. pharm. chim.* [6], 25, 16–26; 378–392. Cf. 384–385 (1907); or Ueber den Nachweis der Glykoside in der Pflanzen mit Hilfe von Emulsin. *Arch. Pharm.*, 245, 172–180. Cf. 173–174 (1907).

Armstrong, H. E., Armstrong, E. F., and Horton, E. Studies on enzyme action. XII.—The enzymes of emulsin. *Proc. Roy. Soc.* (*London*) [B], 80, 321–331. Cf. 324 (1908). The preparation of emulsin from either sweet or bitter almonds is included.

Armstrong, E. F. The rapid detection of emulsin. *J. Physiol.*, 40, XXXII (1910).

Armstrong, H. E., Armstrong, E. F., and Horton, E. Studies on enzyme action. XVI.—The enzymes of emulsin. (1) Prunase, the correlate of prunasin. *Proc. Roy. Soc.* (*London*) [B], 85, 359–362 (1912).

Helferich, B Über Emulsin. *Z. physiol. Chem.*, 117, 159–171. Cf. 166–169 (1921).

Willstätter, R., und Csányi, W. Zur Kenntnis des Emulsins. *Z. physiol. Chem.*, 117, 172–200 (1921).

Josephson, K. Enzymatische Spaltung von Glucosiden. Über die Wirkungsweise der β-Glucosidase des Emulsins. *Z. physiol. Chem.*, 147, 1–154. Cf. 9–15 (1925).

Josephson, K. Enzymatische Synthese von Glucosiden. Über die synthetisierende Wirkung der β-Glucosidase des Emulsins. *Z. physiol. Chem.*, 147, 155–183 (1925).

Expt. 204. An Enzyme Synthesis: β-Methylglucoside.—According to the present conception an enzyme is a biological catalyst which merely accelerates a reaction and in no way determines the direction in which the reaction shall go. Instead the direction in which the reaction proceeds is dependent on the ratio of the concentration of reacting substances to the concentration of reaction products. This theory of the role played by enzymes in biological reactions has been based largely on experiments dealing with syntheses with the protein-hydrolyzing enzyme, pepsin, and the glucoside-splitting enzyme, emulsin.

Place 2 gm. of pure glucose in a 100 cc. volumetric flask and make to volume with 70 per cent, by weight, methyl alcohol. Add 0.6 gm. of the enzyme, emulsin, mix thoroughly and polarize a portion in a 200 mm. polariscope tube at 20° C. Return the contents of the tube to the flask, stopper tightly and allow to stand for 30–35 days at room temperature. At the end of this period withdraw two 5 cc. portions and determine the glucose remaining uncombined (Expt. 139 or 140). Polarize another portion in a 200 mm. tube at 20° C. and note the change in rotation between this and the initial reading. The magnitude of the readings should be about +7 and −1–2° on the Venske scale respectively for the initial and final polarizations. Distill the solution under diminished pressure to remove the alcohol and dissolve the crystalline residue in the least possible amount of hot ethyl acetate. Cool, allow crystallization to take place, filter to remove the crystals of β-methylglucoside and recrystallize these from hot ethyl acetate. Determine the melting-point (Expt. 128). This should be 108–110° C.

From the rotation change during the synthesis of the β-methylglucoside, numerous investigators have calculated that the equilibrium in the reaction has been reached when about 80 per cent of the glucose has been combined. Compare this with the results from the glucose determination.

Van Ekenstein, W. A. Sur le second méthylglucoside. *Rec. trav. chim.*, 13, 183–186 (1894). This author was the first to prepare β-methylglucoside.

Bourquelot, E., et Bridel, M. De l'action hydrolysante et de l'action synthetisante de l'émulsine dans l'alcool méthylique. Obtention du méthylglucoside β. *J. pharm. chim.* [7], 6, 56–62. Cf. 59–60 (1912).

*Bourquelot, E., et Verdon, E. La réversibilité des actions fermentaires: Emulsine et méthylglucoside β. *J. pharm. chim.* [7], 7, 377–383 (1913); or *Compt. rend.*, 156, 957–959 (1913). The latter reference includes the results only.

*Bourquelot, E., et Verdon, E. De l'emploi de proportions croissantes de glucose dans la synthèse biochimique du méthylglucoside β. Influence du produit de la réaction sur l'arrêt de celle-ci. *J. pharm. chim.* [7], 7, 575–579 (1913). This investigation was made with 70 per cent, by weight, of methyl alcohol and using different weights of glucose.

Verdon, E. Contribution à l'étude de l'action synthétisante de l'émulsine. Action de l'émulsine sur le glucose en solution dans l'alcool méthylique; *Thèse doct. Univ.* (*Pharm.*), p. 50, Paris, 1913.

*Hérissey, H. Les glucosides. *Bull. soc. chim.* [4], 33, 349–413. Cf. 385–386, footnote (1923).

Armstrong, E. F. The simple carbohydrate and the glucosides. 4th ed. Longmans, Green & Company, London, 1924.

Wattiez, N. Sur la présence de méthylglucoside β dans les feuilles de *Scabiosa succisa* L. (Dipsacées.) *Bull. soc. chim. biol.*, 7, 917–924 (1925).

Hudson, C. S. The methyl glucosidic derivatives of the sugars. *J. Am. Chem. Soc.*, 47, 268–280 (1925).

IV. Esterases

Expt. 205. Lipase from Castor Beans.—This is prepared from the castor bean (*Ricinus communis*). *Ricin*, the protein of the castor bean, belongs to the toxalbumens and is extremely poisonous. Great care must be taken that no dust from the ground beans is inhaled during the grinding process. Label all bottles with a poison label. Remove the shells from the beans by cracking, and peeling them off by hand; grind the kernels coarsely and extract with ether to remove a large portion of the fat. Mix the meal with enough ether to moisten it thoroughly and allow to stand an hour. Stir occasionally. Then filter on a Büchner funnel using a hardened filter paper. When the ether has ceased dripping through, turn off the suction and wash the meal twice with fresh ether by pouring on enough to wet it thoroughly and cover it; let it begin to drip through before again turning on the suction. Allow the extracted meal to stand in a place out of danger of fire until the ether has evaporated; again grind finely. In order to remove the oil completely, use a Soxhlet apparatus and extract the meal with ether or petroleum ether for a week or more. Keep it at room temperature until only a faint odor of ether remains and then grind to a powder fine enough to pass a 40-mesh sieve.

Green, J. R. On the germination of the seed of the castor-oil plant (*Ricinus communis.*) *Proc. Roy. Soc.*, (*London*) 48, 370–392 (1890). The author discovered vegetable lipase in the germinating seeds of this plant.

*Taylor, A. E. On the action of lipase. *J. Biol. Chem.*, 2, 87–104 (1906–07). The lipase is prepared from castor beans and its properties are carefully studied.

Falk, K. G., and Nelson, J. M. Studies on enzyme action. I. Some experiments with castor bean lipase. *J. Am. Chem. Soc.*, 34, 735–745 (1912).

*Barton, A. W. The lipolytic activity of the castor and soy bean. *J. Am. Chem. Soc.*, 42, 620–632 (1920).

Willstätter, R., und Waldschmidt-Leitz, E. Über Ricinuslipase. *Z. physiol. Chem.*, 134, 161–223 (1924). The authors describe the preparation of lipase cream and defatted seed.

Sandberg, Marta, and Brand, E. On papain lipase. *J. Biol. Chem.*, 64, 59–70 (1925). The lipase estimations were carried out as described by Willstätter, using olive oil as substrate.

Brand, E., and Sandberg, Marta. On the unity of castor lipase. *Proc. Soc. Exptl. Biol. Med.*, 23, 541–544 (1926). "The activated castor bean lipase cream is especially suitable for use in lecture experiments, when it is desirable to demonstrate to a large audience within an hour the *synthesizing* as well as the *hydrolyzing* power of one and the same enzyme preparation."

Expt. 206. Estimation of Lipase Activity.—An emulsion of a fat or oil is the logical substrate for use in lipase studies. An artificial milk made from a lipase-free oil or fat and a gum like acacia furnishes

a suitable emulsion; it also has the advantage of being free from other substances which are likely to yield acids. Palmer has discovered that formaldehyde of about 1 : 1500 is the best bacterial antiseptic available where an artificial milk is used for the substrate. He also points out that neither formaldehyde nor any other antiseptic is suitable for lipase determinations when the substrate is rich in protein and the lipase preparation also contains proteoclastic enzymes. Protein hydrolysis is not prevented entirely by the concentration of formaldehyde used. However, the use of the protein-free artificial milk obviates the difficulty. The small amount of protein material in the lipase preparation is of little importance, since any acidity which it may develop can be obtained in the check which is run at the beginning of the experiment. The hydrolysis of the fat is allowed to come to a natural equilibrium. Barton found that it is necessary to have both the undecomposed fat and the free fatty acids, resulting from the hydrolysis, in solution before titrating. To accomplish this when using an artificial milk, Palmer adds either 100 cc. of acetone-ether (3 : 1) or 150 cc. of 95 per cent ethyl alcohol-ether (1 : 1). The hydrogen-ion concentration of the 4 per cent acacia milk made from any one of several different fats and oils has a pH of 4.9. This milk does not change in reaction on standing and will keep for several weeks before the emulsion breaks. Haley and Lyman found that the optimum hydrogen-ion concentration for castor bean lipase is about pH 5.0.

Add to a 300 cc. Erlenmeyer flask 100 cc. of an artificial milk (Expt. 21), 2 cc. of a 10 per cent suspension of castor bean lipase or 2 cc. of a 6 per cent suspension of steapsin. Add immediately 20 cc. of 0.25–0.3 per cent solution of formalin, containing approximately 40 per cent formaldehyde. Determine the initial acidity at once. Using a pipet, withdraw 25 cc. of the milk and add either 100 cc. of acetone-ether (3 : 1) or 150 cc. of 95 per cent ethyl alcohol-ether (1 : 1). Titrate to a faint permanent pink with 0.1 *N* alcoholic potassium hydroxide solution, using 5 drops of 1 per cent phenolphthalein solution as indicator. Incubate the remainder of the milk for 24 hours at 38° C. with occasional rotation of the flask, and repeat the titration on another 25 cc. aliquot. Calculate the amount of 0.1 *N* acid developed.

Alcoholic potassium hydroxide solution.—See Experiment 180.

Palmer, L. S. The preservation of milk for chemical analysis. *Missouri Agr. Expt. Sta. Res. Bull.*, 34, 1919.

Rector, T. M. Lipolytic enzymes in olive oil. *J. Ind. Eng. Chem.*, 12, 156–158 (1920).

*Barton, A. W. The lipolytic activity of castor and soy beans. *J. Am. Chem. Soc.* 42, 620–632 (1920).

*Haley, D. E., and Lyman, L. E. Castor bean lipase, its preparation and some of its properties. *J. Am. Chem. Soc.*, 43, 2664–2670 (1921).

*Palmer, L. S. The influence of various antiseptics on the activity of lipase. *J. Am. Chem. Soc.*, 44, 1527–1538 (1922).

V. Oxidases

Expt. 207. Artificial Auto-oxidation.—According to Engler and his co-workers every oxidation consists primarily of the union of molecular oxygen with the substance to be oxidized. The primary product of this is a peroxide of the substance undergoing oxidation. This peroxide resulting from the auto-oxidation of the carrier, brings about the oxidation of the second substance, the acceptor. Thus, when an auto-oxidizable substance, A, is in contact with oxygen and a second oxidizable substance, B, the following changes take place:

$$A + O_2 = AO_2$$

$$AO_2 + B = AO + BO$$

In this way a substance incapable of combining directly with oxygen may be oxidized through the intervention of another substance, and a given quantity of oxygen will be equally distributed between them. This method of auto-oxidation leads very readily to the present conception of oxidizing enzymes and their action. The following experiments illustrate the principles of auto-oxidation:

(*a*) *With indigo.*—Place 10 cc. of an indigo carmine solution in a test tube, add 1 to 2 cc. of benzaldehyde and shake vigorously. Allow to stand for several hours and shake at intervals. Explain the chemical changes that have taken place by means of equations. What substance is the acceptor?

Indigo carmine solution.—Dissolve 0.01 gm. of indigo carmine in 500 cc. of distilled water.

*Baeyer, A., und Villiger, V. Benzoylwasserstoffsuperoxyd und die Oxydation des Benzaldehyde an der Luft. *Ber.*, 33, 1569–1585 (1900).

(*b*) *With guaiacum.*—Place 5 cc. of purified gum guaiacum solution in a test tube, add 1 cc. of benzaldehyde and shake vigorously. Allow to stand for several hours and shake at intervals. Explain the result.

Guaiacum solution.—Dissolve 2 gm. of gum guaiacum in 100 cc. of 95 per cent ethyl alcohol. Boil this solution with animal charcoal to remove the peroxides, which may have been formed by auto-oxidation of the guaiacum.

Richter, P. Zur Kenntnis des Guajakharzes. *Arch. Pharm.*, 244, 90–119. Cf. 113–119 (1906). The author believes that guaiaconic acid, which is the active constituent of the gum, gives guaiac-blue on oxidation.

Lyle, W. G., and Curtman, L. J. A new method for the detection of occult blood in stools. *J. Biol. Chem.*, 33, 1–6 (1918). A description of the preparation of guaiaconic acid from gum guaiacum is included.

(*c*) *With potassium iodide.*—Mix 5 cc. of potassium iodide solution with a few drops of a soluble starch solution. Add about 1 cc. of benzaldehyde and shake thoroughly. Allow to stand several hours, shaking at intervals. Explain the changes that take place.

Potassium iodide solution.—For the preparation of this solution see Experiment 184.

(*d*) *With indigo.*—Place 10 cc. of indigo carmine solution in a test tube, add 1–2 cc. of old turpentine and shake vigorously. Allow to stand for several hours, shaking vigorously at intervals. Explain the changes that take place.

Traube, M. Ueber Aktivirung des Sauerstoffs. *Ber.*, 15, 659–675 (1882).

*Engler, C., und Wild, W. Ueber die sogenannte "Activirung" des Sauerstoffs und über Superoxydbildung. *Ber.*, 30, 1669–1681 (1897).

*Engler, C., und Weissberg. Kritische Studien über die Vorgänge der Autoxydation. Braunschweig. 1904.

Expt. 208. Oxidase Reactions of a Plant Sap.—It is well known that the tissues of many plants discolor or turn brown when injured or bruised. It has been also shown that the expressed juices or water extracts of these plants produce a blue color when treated with a gum guaiacum solution in the presence of air. On the other hand, there will will be no blue color in the expressed juices or water extracts of those plants which do not discolor on injury, until hydrogen peroxide has been added with the guaiacum solution. The Bach and Chodat hypothesis of oxidizing enzymes, which is the one generally accepted, holds that they consist of two components, an oxygenase or direct oxidase and a peroxidase or indirect oxidase. When a plant tissue, extract or juice gives a blue color with guaiacum solution it is said to contain a direct oxidizing enzyme. If, however, hydrogen peroxide has to be added before a blue color develops, an indirect oxidizing enzyme or peroxidase is present. In general, a system which turns guaiacum blue contains a peroxide and a peroxidase. The peroxide may be either some organic peroxide or hydrogen peroxide. The peroxidase acts upon the peroxide and deprives it of an atom of oxygen which brings about the oxidation of a second substance, the acceptor. Guaiacum is the acceptor in this discussion. In the case of a direct reaction it is believed that some organic substance acts as a peroxide, while in the indirect reaction the peroxidase only is present, and a peroxide, such as hydrogen peroxide has to be added. In testing for oxidizing enzymes the divergent results obtained by various investigators depend upon whether tests were

made on fresh tissue or upon extracts or juices. It has been found that the maximum activity of the tissue is usually shown when it is placed directly in the reagent. However, it is advisable to examine the materials in both ways.

Grind a cabbage (*Brassica oleracea*) through a food chopper, after first removing the outer green leaves; then press out the juice. Put a peeled potato tuber (*Solanum tuberosum*) through a food chopper using the nut butter attachment, or grate it on a fine vegetable grater. Grind the macerated tissue in a mortar with a small quantity of distilled water and, after allowing it to stand for 15 minutes, filter. Scrape off a very thin layer of the outer portion of a potato tuber and make a water extract. Clear yellowish-brown solutions are obtained from the potato pulp and scrapings. Observe the extracts of the potato pulp and scrapings for the rapidity of their oxidase reactions. Using portions of the plant sap or extracts, perform the following tests:

(*a*) *Guaiacum reaction.*—To 5 cc. add 10 drops of guaiacum solution (Expt. 207). Note that a blue color develops. For control purposes, test a thoroughly boiled solution in the same manner.

(*b*) *Röhmann-Spitzer reaction.*—To 2 cc. add 5 drops of the indophenol reagent. Observe and record the colors developed in one-half minute and in 15 minutes. These vary from pink to deep reddish-purple. The synthesis of indophenol is brought about by the oxidation of α-naphthol and p-phenylenediamine hydrochloride. This reagent is more delicate than guaiacum or α-naphthol solutions, and its color is more easily detected through the natural color of the juice.

Indophenol reagent.—Mix equal parts of (1) and (2) just before using.

(1) α-naphthol solution.—Prepare a 1 per cent solution of α-naphthol in 50 per cent ethyl alcohol. Upon oxidation a lilac color results.

(2) p-phenylenediamine hydrochloride.—Prepare a 1 per cent solution of p-phenylenediamine hydrochloride in distilled water.

*Röhmann, E., und Spitzer, W. Ueber Oxydationswirkungen thierische Gewebe. *Ber.*, 28, 567–572 (1895).

(*c*) *Benzidene reaction.*—To 5 cc. add 10 drops of a 1 per cent solution of benzidene in 50 per cent ethyl alcohol. Allow to stand half an hour. A blue color develops.

(*d*) *Phenolphthalin reaction.*—To 10 cc. add 5 cc. of the phenolphthalin solution. Allow to stand 30 minutes, then make alkaline with 0.05 N sodium hydroxide solution. The presence of oxidases is shown by the pink or red color of the phenolphthalein, produced by the oxidation of the colorless phenolphthalin.

Phenolphthalin solution.—To 1 cc. of 0.1 *N* sodium hydroxide solution, add 0.1 gm. of phenolphthalin; this is somewhat more than will dissolve in the given quantity of sodium hydroxide. Shake to dissolve as thoroughly as possible, add 25 cc. of distilled water, filter, and dilute the filtrate to 100 cc. A colorless solution of the sodium salt of phenolphthalin results.

Kastle, J. H., and Shedd, O. M. Phenolphthalin as a reagent for the oxidizing ferments. *Am. Chem. J.*, 26, 526–539 (1901).

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*Falk, K. H., McGuire, Grace, and Blount, Eugenia. Studies on enzyme action. XVII. The oxidase, peroxidase, catalase, and amylase of fresh and dehydrated vegetables. *J. Biol. Chem.*, 38, 229–244 (1919).

Expt. 209. Peroxidase Reactions of a Plant Sap.—With portions of the plant sap, which was used in Experiment 208, perform the following tests:

(*a*) *Guaiacum reaction.*—To 5 cc. add 10 drops of guaiacum solution (Expt. 207) and 5 drops of 1 per cent hydrogen peroxide. If peroxidase is present in the plant sap, a more *intense* coloration than was produced in the corresponding oxidase reaction results.

(*b*) *α-naphthol reaction.*—To 2 cc. add 5 drops of α-naphthol solution (Expt. 208*b*) and 2 cc. of 3 per cent hydrogen peroxide. Observe and record the colors developed in one-half minute and in 15 minutes. A pink, a lavender, a deeper lavendar, or a deep bluish-purple color results according to the plant sap used.

(*c*) *Benzidene reaction.*—To 5 cc. add 10 drops of benzidene solution (Expt. 208*c*) and 5 drops of 1 per cent hydrogen peroxide. Observe that a blue color quickly develops; this changes to a dark brown.

(*d*) *Phenolphthalin reaction.*—To 5 cc. add 2.5 cc. of phenolphthalin solution (Expt. 208*d*) and 5 drops of 1 per cent hydrogen peroxide solution. Allow to stand half an hour, then make alkaline with 0.05 *N* sodium hydroxide solution.

Expt. 210. Localization of oxidase and Peroxidase in Plant Tissue. —Make four thin cross sections of an apple (*Pyrus malus*), through the carpillary region and place them on four separate watch glasses. Moisten the freshly cut surface of each with oxidase (Expt. 208) and peroxidase (Expt. 209) reagents, as follows:

(*a*) Benzidene solution

(*b*) Benzidene solution and 1 per cent hydrogen peroxide solution

(*c*) Guaiacum solution

(*d*) Guaiacum solution and 1 per cent hydrogen peroxide solution. Draw a diagram of the apple surface, indicating the location of the largest amounts of oxidase and peroxidase as indicated by the tests. Where do the enzymes seem to be localized?

In a similar manner, make a study of cross sections of a potato tuber (*Solanum tuberosum*).

Place cross sections of both apple and potato on watch glasses, moisten with distilled water, and allow to remain for half an hour. Is there any coloration? If so, explain why.

Expt. 211. Determination of Peroxidase in a Plant Sap.—The method for the quantitative determination of peroxidase, originally suggested by Bach and Chodat, is based upon the oxidation of pyrogallol in the presence of peroxidase and hydrogen peroxide. The peroxidase liberates atomic oxygen from the hydrogen peroxide, and this in turn reacts with pyrogallol to form insoluble purpurogallin. It is evident that the end product of the peroxidase action can not be measured directly, but must be measured indirectly by its action on pyrogallol.

Prepare a plant sap (Expt. 66) using either cabbage (*Brassica oleracea*), rutabagas (*Brassica oleracea*, var. *Napo-brassica*), white turnips (*Brassica rapa*), carrots (*Daucus carota*), or potato tubers (*Solanum tuberosum*). Place in each of two 250 cc. Erlenmeyer flasks the following solutions in the order named: 65 cc. of freshly boiled distilled water, 20 cc. of freshly prepared 5 per cent pyrogallol solution, and 20 cc. of freshly prepared 1 per cent hydrogen peroxide solution. The distilled water must be boiled to remove the carbon dioxide, which affects the rate of peroxidase activity. Cool the contents of the flasks in an ice mixture to about 10° C., then pipet into each 5 cc. of the plant sap containing the peroxidase, shaking during the process. Count the time for beginning the experiment at the instant the pipet is half discharged. For example, if a pipet discharges in 20 seconds, the experiment begins at the end of 10 seconds. At the end of 10 minutes, pipet into each flask 5 cc. of 2 *N* hydrochloric acid to inhibit the activity of the peroxidase. Filter off the red purpurogallin precipitate on previously prepared and weighed Gooch crucibles (Expt. 139). Wash the

precipitates with a solution made by mixing 30 cc. of distilled water with 10 cc. of 5 per cent pyrogallol solution and 10 cc. of 1 per cent hydrogen peroxide solution cooled to about 10° C. To remove the pyrogallol wash with very small quantities of distilled water until the washings no longer show a dark blue color when tested with a few drops of ferric chloride. Use the least possible amount of distilled water, for purpurogallin is slightly soluble. Dry the crucible at 100° C. for one hour, cool in a desiccator and weigh. Reheat to constant weight.

The weight in milligrams of purpurogallin, yielded by 5 cc. of a plant sap at its normal hydrogen-ion concentration is the *peroxidase number* of that plant sap. Plant saps which contain an extremely small amount of peroxidase will not give sufficient purpurogallin to form a precipitate and may not therefore be tested by this method. Under such conditions the purpurogallin should be extracted with ether and estimated colorimetrically using an ether solution as a standard according to the method of Willstätter and Stoll.

*Bach, A., und Chodat, R. Untersuchungen über die Rolle der Peroxyde in der Chemie der lebenden Zelle. VIII. Ueber die Wirkungsweise der Peroxydase. *Ber.*, 37, 1342–1348 (1904).

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Willstätter, R., und Pollinger, A. Peroxydase. *Ann.*, 430, 269–319 (1923).

Harvey, E. H. The pH value of some common foods. *Am. Food Jour.*, 19, 560 (1924).

Expt. 212. Separation of Peroxidase and of Aromatic Substances from Potato Tubers.—*Components of the oxidase system according to Onslow.*—Onslow has recently developed a system of oxidizing enzymes which differs from that of Bach and Chodat. She has observed that those plants which give a blue color with guaiacum solution contain an aromatic substance with the ortho-dihydroxy grouping, in addition to a peroxidase. In those plants in which the aromatic substances are lacking, the bluing of guaiacum is brought about only after the addition of hydrogen peroxide. In the first type of plants, the tissue can be freed from the aromatic substances by pounding and extracting in cold ethyl

alcohol. This precipitates the enzymes of the cells and retains them in the tissue residue, at the same time removing other substances such as sugars, tannins, and organic acids, which may act as inhibitors. A water extract of this tissue residue treated with guaiacum will become blue only on the addition of hydrogen peroxide. This indicates that the extract contains peroxidase alone. When this tissue extract is added to a solution of aromatic substances, oxidation takes place with the formation of a brown color which indicates the production of a peroxide. The resulting combination, peroxide and peroxidase, will then blue guaiacum. Thus two reactions are catalyzed by the water extract of the tissue residue. One is the oxidation of the aromatic substance by means of molecular oxygen with the formation of a peroxide; the other, the decomposition of the peroxide by means of the peroxidase.

This author has suggested that a second enzyme, in addition to the peroxidase, is present in those plants which contain an ortho-dihydroxy grouping. The function of this additional enzyme is to catalyze the oxidation of the aromatic substance by the formation of a peroxide, which when formed, is acted upon by the peroxidase in the usual way. For this second enzyme she has proposed the name oxygenase. Thus three factors are necessary for the bluing of guaiacum: first, an aromatic substance with the ortho-dihydroxy grouping; second, an enzyme, oxygenase, which catalyses the auto-oxidation of such substances with the production of an organic peroxide; and third, a peroxidase.

The following experiments illustrate the separation of these components.

(*a*) *Separation of peroxidase.*—For this purpose use either the potato tuber (*Solanum tuberosum*) or the fruit of the pear (*Pyrus communis*). The pear is better than the potato because the latter contains, in addition to a peroxidase, a tyrosinase and a substance generally considered to be tyrosine, which this oxidizes. Most of the darkening of expressed potato juice is due to the presence of tyrosinase. If pears are employed, a variety which browns rapidly on injury should be selected.

Rapidly cut a peeled potato or pear into thin slices, placing these in a mortar which contains 95 per cent ethyl alcohol, then pound thoroughly. The alcohol prevents the exposure of the tissue to the air. Filter off the alcohol quickly on a Büchner funnel. Again grind the residue with more alcohol and filter. Repeat the process two or three times. Filter off the final residue and dry. The white powder contains not only peroxidase and oxygenase but also tyrosinase. Save a portion for the estimation of peroxidase (Expt. 211), and for tyrosinase tests (Expt. 213). Prepare a cold water extract of some of this tissue residue and filter. Use portions of the solution for the following tests:

1. Allow one portion of the solution to stand exposed to the air for an hour or two. Does the solution darken?

2. To another portion add guaiacum solution. Does a blue color develop within an hour?

3. To another portion add both guaiacum solution and hydrogen peroxide. A deep blue color is obtained. Explain the result.

(*b*) *Extraction of aromatic substances.*—Peel several potatoes or pears, cut them into thin slices as rapidly as possible and drop them immediately into an Erlenmeyer flask containing boiling 95 per cent ethyl alcohol. Boil the contents of the flask under a reflux condenser for half an hour, and then filter off the alcoholic extract while hot. Concentrate the filtrate in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) until all the alcohol is removed. The residue is a turbid aqueous solution consisting of those substances which have been extracted by the alcohol, and of water which is largely derived from the tissue of the potato tubers or pears. Filter and add to the filtrate just enough of a 15 per cent solution of neutral lead acetate to bring about complete precipitation. The precipitate consists chiefly of the salts of aromatic substances and organic acids. Filter off the precipitate and wash it with distilled water. Decompose it with a minimum amount of a 5–10 per cent solution of sulfuric acid and filter off the lead sulfate. Neutralize the filtrate to litmus with sodium hydroxide solution and use portions for the following tests:

1. To one portion add a few drops of 4 per cent ferric chloride solution, avoiding an excess. A green color is produced. On the addition of a few drops of a 1 per cent sodium carbonate solution, the green color changes to a blue, purple or red. The reaction with ferric chloride and sodium carbonate is characteristic of catechol, protocatechuic acid, protocatechuic aldehyde and caffeic acid, all of which substances contain the ortho-dihydroxy grouping. This reaction may be referred to as the " catechol " reaction.

2. To another portion of the solution add an aqueous extract of the peroxidase prepared in (*a*). Allow to stand about an hour and observe. Explain this result.

3. Remove a portion of solution (2) any time within an hour after the addition of the peroxidase and add guaiacum tincture. A blue color is obtained. This shows the presence of oxygenase.

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Willstätter, R., und Pollinger, A. Peroxydase. *Ann.*, 430, 269–319 (1923).

Expt. 213. Soluble and Insoluble Tyrosinase from Meal Worms.—Grind 100 of the larva or about 12.5 gm. of the yellow meal worm (*Tenebrio molitor Linn.*) in a large mortar with 150–175 cc. of distilled water, saturated with chloroform. Strain the milky liquid through two or three thicknesses of cheese cloth, and preserve it in a flask closed with a stopper, for it rapidly darkens at the surface when in contact with the air. Grind the residue with a small quantity of chloroform-water, again strain and repeat the process until the filtrate is no longer milky.

Soluble tyrosinase.—Filter the combined extracts on porous filter paper, covering with a watch glass; collect the filtrate in a beaker, containing powdered ammonium sulfate in excess of the amount required to produce a saturated solution. The soluble tyrosinase and the colloidal insoluble tyrosinase are coagulated together in the filtrate, as a light gray mass. Use the residue for the preparation of insoluble tyrosinase. Filter; wash the coagulum with saturated ammonium sulfate solution, and dissolve it as completely as possible in distilled water. Filter this solution and saturate the filtrate with powdered ammonium sulfate; again filter; wash the coagulum with saturated ammonium sulfate solution; dissolve it in 20–25 cc. of 0.05 per cent anhydrous sodium carbonate solution, and filter. This filtrate contains the *soluble* tyrosinase. To 10 cc. of a saturated tyrosine (Expt. 103) solution, add 1.5 cc. of the filtrate, containing the soluble tyrosinase; mix thoroughly, and allow to stand 24 hours. The solution passes through a series of color changes, ranging from pink to rose, to red, to violet, to blue-black, and finally a black precipitate is formed. In what part of the solution does the coloration begin? Explain the color changes produced by tyrosinase.

Insoluble tyrosinase.—Wash the residue from the original filtration of the extract with chloroform-water for several days. Then transfer it to a small flask, mix thoroughly with 25 cc. of chloroform-water, allow to stand over night and filter through double *barium* filter papers. Mix 10 cc. of this filtrate with 10 cc. of a saturated tyrosine solution and allow to stand for 24 hours. If at the end of this time no color has developed, the soluble tyrosinase has been completely removed and the

residue contains the very active insoluble tyrosinase. Place this residue in a test tube containing sufficient chloroform and distilled water (1 : 4) to cover it completely. Preserved thus, it may be kept for several months without diminution of its activity.

To 10 cc. of saturated tyrosine solution add a few drops of the suspension and allow to stand for 24 hours. The solution passes through a series of color changes until finally a black precipitate is formed, leaving a clear supernatant liquid. Record the color changes. Remove the supernatant liquid, add fresh tyrosine solution and again observe and record the color changes. To indicate the activity of the insoluble tyrosinase, these steps may be repeated several times with the same sample.

Again add a few drops of the suspension of insoluble tyrosinase to 15 cc. of saturated tyrosine solution, and allow to stand until a deep rose color has developed. Then filter one-half of the mixture twice through *barium* filter paper to remove the enzyme. Place this filtrate, and the unfiltered portion in the dark for 24 hours, but observe from time to time for any changes in color. Preserve the filtered portion for a week and observe.

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VI. Catalase

Expt. 214. The Detection and Estimation of Catalase.—The enzyme, catalase, was first extensively studied by Loew, although Schoenbein was apparently the first to show that extracts of vegetable and animal tissue were capable of decomposing hydrogen peroxide with the evolution of oxygen. Later studies indicate that catalase may be intimately associated with the phenomena of cell respiration, therefore the detection and estimation of catalase activity becomes of primary importance.

Detection.—If the "pressaft" from most fresh plant or animal tissues is added to a neutral solution of hydrogen peroxide, oxygen is rapidly liberated as may be seen by the formation of numerous small bubbles or, if the reaction is carried out in a test tube, the oxygen may be detected by the usual glowing pine splinter method.

Estimation.—The estimation of the intensity of catalase activity is carried out by (*a*) measuring the volume of oxygen liberated from hydrogen peroxide in contact with a tissue extract in a given period of time or (*b*) better, by measuring the time which is required for a given amount of tissue extract to liberate one-half of the available oxygen from a given amount of hydrogen peroxide.

A satisfactory apparatus for the measurement may be constructed from a wide-mouthed 250 cc. bottle fitted with a 3-hole rubber stopper. Through one hole is inserted a small separatory funnel, the second hole admits a glass stop-cock, and the third hole a glass gas-delivery tube which is connected through a flexible rubber connection to the inlet tube of a 100 cc. Hempel-Winkler gas buret; or if the gas buret is not available, the delivery tube may extend below the surface of the water of a pneumatic trough and into an inverted 100 cc. graduate cylinder filled with water.

Grind a portion of plant tissue or of a potato tuber with clean quartz sand and a small amount of pure precipitated calcium carbonate in a mortar until it is completely macerated. Squeeze the pulp in a fold of clean linen or muslin, collecting the juice in a beaker, or use sap extracted as in Experiment 66. Place 2 5 cc. of the juice in a small glass container. For this a piece of glass tubing with a thin-walled bulb blown on one end is satisfactory. Insert this in the 250 cc. bottle so that

there is no danger of the juice being spilled, should the apparatus be jarred. Now connect the bottle with the remainder of the apparatus and immerse it in a constant temperature thermostat at 20° C. Add through the separatory funnel, standard neutral hydrogen peroxide solution equivalent to 80 cc. of oxygen. The hydrogen peroxide may be standardized by titration with standard potassium permanganate solution. Add sufficient water to bring the total volume of liquid in the bottle to approximately 50 cc. After temperature equilibrium has been reached, adjust the water level in the gas buret by means of the glass stop-cock in the reaction bottle. Then close the stop-cock, lower the water level, and start the reaction by shaking the reaction bottle sufficiently hard to break the thin glass bulb containing the catalase preparation; this permits the enzyme solution to mix with the peroxide. Note this time exactly. Oxygen will now be freely evolved. Continue shaking the reaction chamber and note the time at which exactly 40 cc. of oxygen has been liberated. The length of time required for the catalase preparation to liberate the 40 cc. of oxygen is a measure of the comparative amount of catalase in the solution. Catalase has its maximum activity at approximately pH = 7.0 and is readily inactivated by heat.

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I. The effect of different hydrogen peroxide concentrations.

II. Loss of catalase activity.

III. Temperature effect at different hydrogen ion concentrations.

IV. A theory of the catalase reaction.

J. Biol. Chem., 68, 521–563 (1926).

VII. Applications

Expt. 215. Tests for the Presence of Enzymes in Sprouted Grain.—Sprout 300 gm. of wheat or corn by exposing it to a humid atmosphere

in a water oven at 40° C. for several days. When the sprouts are about 1 cm. long, remove the seeds, spread in a thin layer, and dry in the air blast of an electric fan or in a ventilated drying oven at 40° C. Grind to a coarse meal that will pass through a 20-mesh sieve. At no time in the germinating or drying processes should the temperature be allowed to rise above 50° C. Stir the meal into sufficient distilled water to form a thin paste; extract by grinding in a mortar or in a ball mill for 2–3 hours. Filter or centrifuge and preserve the aqueous enzyme extract under toluene.

Using portions of the filtrate, test for the presence of individual enzymes according to Experiments 191, 192, 194, 195, 200, 206, 208, 209, and 214.

Bach, A., und Oparin, A. Über die Fermentbildung in keimenden Pflanzensamen. *Biochem. Z.*, 134, 183–193 (1923). A scheme for the quantitative estimation of the enzymes of sprouting seeds is given in this paper.

CHAPTER IX

PLANT PIGMENTS

I. Chlorophyll and Carotinoid Pigments

Expt. 216. Extraction of Chlorophylls *a* and *b*, Carotin, and Xanthophyll.—*Preparation of leaf material.*—Collect leaves from vigorous plants during the spring and early summer at a time when they are not wet by dew or rain. It is essential that the leaves be dried rapidly and without exposure to direct sunlight. Immediately place them on trays with wire bottoms to permit free circulation of air, and dry in a drying oven, having steam coils, and heated to 30–40° C. The doors may be left open and an electric fan used to hasten the drying which should be accomplished in from 18 to 24 hours. When the leaves are dry, place them in a ball mill and grind to a powder. Since the carotinoid pigments oxidize very readily, that portion of the powder not used immediately should be stored in tin- or glass-sealed containers away from the light. Leaf material may be kept in this manner for several years and used as desired.

Extraction of the chloroplast pigments.—Place 4 gm. of leaf powder of either the stinging nettle (*Urtica dioica*) or the cow pea (*Vigna sinensis*) in a 5 cm. Büchner funnel, fitted with a filter paper. Add a small quantity of 85 per cent acetone; allow it to soak into the meal for a few minutes; then apply suction. Repeat this operation until 40 cc. of the acetone has been added. A deep blue-green solution which contains all four chloroplast pigments, results. Observe the solution in both transmitted and reflected light. Transfer the pigments from the acetone extract to petroleum ether by pouring 20 cc. of the extract into twice its volume of *petroleum ether*, contained in a small separatory funnel. Add a volume of distilled water, equal to the combined volumes of the acetone extract and petroleum ether; pour it gently down the side of the funnel to avoid the formation of emulsions. In a few minutes the petroleum ether layer containing the pigments separates; draw off and discard the lower layer which is slightly green. To remove completely the acetone from the petroleum ether solution, wash it four times, using

an equal volume of distilled water and drawing off the lower layer after each washing. Use this petroleum ether solution in Experiment 217.

Prepare an *ether* solution by transferring 20 cc. of the acetone solution containing the pigments to pure ether, using the method by which it was transferred to petroleum ether. The ether solution will be used in Experiments 217, 218, 219, and 220, but it should not be prepared until it is needed.

Willstätter, R. Chlorophyll. *J. Am. Chem. Soc.*, 37, 323–345 (1915).

*Jörgensen, I., and Stiles, W. Carbon assimilation. A review of recent work on the pigments of the green leaf and the processes connected with them. E. Simple laboratory experiments on the leaf pigments. *New Phytologist*, 15, 11–23 (1916). One of a series of articles on carbon assimilation. Suggestions for Experiments 216–222 inclusive are also taken from this reference.

Expt. 217. Demonstration of the Presence of Chlorophylls *a* and *b*. Place 20 cc. of a petroleum ether solution of the mixed pigments (Expt. 216), and 20 cc. of 92 per cent methyl alcohol in a separatory funnel. Shake thoroughly and then allow to stand. Two layers separate; the upper or petroleum ether layer containing chlorophyll *a*, is blue-green; while the lower or methyl alcohol layer, containing chlorophyll *b*, is green. The color difference between them is less distinct because of the presence of the yellow pigments of which carotin is in the petroleum ether, and xanthophyll in the methyl alcohol. Preserve these solutions for use in Experiment 218.

Expt. 218. Saponification of Chlorophylls *a* and *b*: The Phase Test.—Pour 10 cc. of an ether solution of the mixed pigments (Expt. 216) into a test tube. Place the lower end of a pipet, containing a few cubic centimeters of fresh 30 per cent acetone-free methyl alcoholic potassium hydroxide solution at the bottom of the test tube and allow the alkali solution to run in below the pigment solution. At the interface between the solutions there appears immediately a dark-brown layer, the so-called brown phase which diffuses on shaking. In about 10 minutes it changes back through an olive-green to pure green. This indicates that the chlorophylls have been saponified in the *cold* alkali, forming the potassium salts of the acids, chlorophyllin *a* and chlorophyllin *b*. These salts are insoluble in ether, but soluble in water. Add distilled water and shake; upon standing two layers separate: a water layer which is green but not fluorescent, and an ether layer which is practically colorless. The brown phase which appears when saponification occurs is the result of a yellow phase produced by chlorophyll *a* and a brown-red phase produced by chlorophyll *b*.

Demonstrate this by the phase test on the two chlorophylls separately. To do it transfer the methyl alcohol solution containing

chlorophyll *b* (Expt. 217) to ether (Expt. 216). Saponify the latter solution and the petroleum ether solution containing chlorophyll *a* (Expt. 217) according to the method just outlined.

Expt. 219. Separation of Green from Yellow Pigments.—Place 10 cc. of an ether solution of the mixed pigments (Expt. 216) in a test tube, add 4 cc. of *fresh* 30 per cent acetone-free methyl alcoholic potassium hydroxide solution and shake. After the green color has reappeared following the brown phase (Expt. 218), slowly add 20 cc. of distilled water and a little more ether. Shake the test tube and allow to stand. Two layers separate: the lower or alkaline contains the saponified chlorophylls *a* and *b* and is green; while the upper or ether layer contains the carotin and xanthophyll and is yellow. Wash the ether solution of yellow pigments with distilled water in a separatory funnel and preserve for the succeeding experiment.

This test may be used to determine the purity of a chlorophyll preparation. If the yellow pigments have been entirely removed, the ether layer should remain colorless after the saponification of the chlorophylls.

Expt. 220. Separation of Carotin and Xanthophyll.—Evaporate the ether solution of yellow pigments (Expt. 219) to 1 c.c. on a water bath. Transfer to a separatory funnel with 20 cc. of petroleum ether, add 20 cc. of 90 per cent methyl alcohol and shake. In a short time two layers separate. Draw off the lower or methyl alcohol layer which contains the xanthophyll, and make successive extractions of the petroleum ether layer with methyl alcohol until the latter is no longer colored. The petroleum ether retains the carotin.

Expt. 221. Substitution of Copper for Magnesium in Chlorophylls *a* and *b*.—Place 10 cc. of an ether solution of the mixed pigments (Expt. 216) and a small quantity of 20 per cent hydrochloric acid in a separatory funnel. Shake thoroughly and then wash with distilled water. The hydrochloric acid acts on both chlorophylls *a* and *b* splitting off the magnesium which is quantitatively replaced by hydrogen; this results in the formation of magnesium-free derivatives, known as phaeophytins. Evaporate the ether solution of the phaeophytins on a water bath to dryness and dissolve the residue in 10 cc. of 95 per cent ethyl alcohol. The solution is olive-green in color. Add a particle of copper acetate and heat the mixture on a water bath. The olive-green color gradually disappears and finally the solution takes on an intense green color. If the chlorophylls have been completely converted into phaeophytins, there will be no fluorescence in the solution. These copper compounds of the chlorophylls are very similar to the magnesium compounds, but are much more stable.

Woodman, H. E. The nature of the pigment of silage. *J. Agr. Sci.*, 13, 240–242 (1923). Phaeophytin is shown to be the pigment in silage which would therefore be an excellent source for its preparation on a large scale.

Expt. 222. Formation of Phytochlorin *e* and Phytorhodin *g*.—Place 10 cc. of an ether solution of the mixed pigments (Expt. 216) in a test tube and evaporate to dryness on a water bath. Treat the warm residue with 6 cc. of boiling 30 per cent acetone-free methyl alcoholic potassium hydroxide solution and boil gently for half a minute. Saponification of the chlorophylls with *hot* alkali produces the potassium salts of the acids, isochlorophyllin *a* and isochlorophyllin *b*, which have a red fluorescence. The hot alkali also destroys the yellow pigments. To demonstrate this, dilute the solution with twice its volume of distilled water; place in a separatory funnel, and shake with ether. Observe that the ether layer remains colorless. Just acidify the mixture with concentrated hydrochloric acid, shaking thoroughly. Draw off and discard the lower layer. The ether becomes olive-brown in color because of the presence of the magnesium-free isochlorophyllins, phytochlorin *e* and phytorhodin *g*, which are produced by the action of the acid on the isochlorophyllins formed by the alkali treatment. Phytochlorin *e* and phytorhodin *g* may be extracted separately from the ether solution by the use of a specific concentration of hydrochloric acid for each. In each case, one compound is dissolved by the acid, leaving the other in the ether. Shake the ether solution twice with 20 cc. of 4 per cent hydrochloric acid, and draw off the acid layer. Neutralize this blue-green acid solution with ammonium hydroxide, and extract it with ether. The ether solution contains the derivative of chlorophyll *a*, phytochlorin *e*, which gives it an olive-green color. Again extract twice the ether remaining in the funnel after the removal of the blue-green acid solution, using 20 cc. of 12 per cent hydrochloric acid. Dilute the resulting green acid solution with distilled water and extract it with ether. This ether solution contains the derivative of chlorophyll *b*, phytorhodin *g*, which gives it a red color.

Expt. 223. Some Physical and Chemical Properties of Carotin and Xanthophyll.—The solvents used for the extraction of the carotinoid and chlorophyll pigments from fresh or dried plant tissue have been classified by Tswett into three groups based upon the relative solubility of the pigments in each.

1. Methyl, ethyl, and amyl alcohols, acetone, acetaldehyde, ether, and chloroform. All the pigments are equally and completely dissolved from either fresh or dried leaves by these solvents.

2. Petroleum ether. Carotin and traces of the other pigments are more or less completely removed from fresh leaves by this solvent. The dried leaves yield carotin in purer form.

3. Benzene, xylene, toluene, and carbon disulfide. These solvents act intermediately between group 1 and group 2.

From later work done by Willstätter and Mieg and by Tswett a further classification of solvents for extracting carotin and xanthophyll has been made.

1. If a petroleum ether solution of the carotinoid pigments is shaken with either 85 per cent ethyl alcohol or 92 per cent methyl alcohol, the petroleum ether will retain the carotin while the xanthopyll will be extracted by the alcohol.

2. If an 85 per cent ethyl alcohol or a 92 per cent methyl alcohol solution of carotin is shaken with carbon disulfide, the latter will extract the carotin. Under similar conditions xanthophyll will be distributed between both solvents.

Solubility.—The solubility of pure xanthophyll and pure carotin in a few solvents at 25° C. as determined by Schertz is given in Table XIV.

TABLE XIV

Solvent	Milligrams per Liter	
	Xanthophyll	Carotin
Petroleum ether.....	9.5	626.0
Absolute ethyl alcohol....	201.5	15.5
Absolute methyl alcohol..	134.9	Nearly insoluble
Pure ether (anhydrous)...	952.0	1005.0

Observe that carotin and xanthophyll have nearly the same solubility in pure ether; that xanthophyll is 13 times as soluble as carotin in absolute ethyl alcohol; while in petroleum ether about 66 times as much carotin as xanthophyll is dissolved showing that in this solvent there is the most difference in the solubility of the pigments. Palmer points out that while pure xanthophyll is almost insoluble in petroleum ether, when in the amorphous state or contaminated with lipoids, it dissolves quite easily in this solvent.

Color.—(*a*) Carotin. Very dilute solutions of carotin in ether, petroleum ether, or ethyl alcohol are yellow; more concentrated solutions are deep orange; and very concentrated, deep red except in the case of alcohol when a saturated solution is yellow. A dilute solution of carotin in carbon disulfide is pink to red; while the more concentrated solutions are red, or dark red to almost black.

(*b*) Xanthophyll. The solution of xanthophyll in any of the common solvents, except carbon disulfide is yellow; but in the latter it is orange to orange-red. The xanthophyll solutions may be distinguished from the corresponding carotin solutions by the greenish tinge which they display when much diluted.

Stability.—Absolute ethyl alcohol and petroleum ether solutions of pure carotin and pure xanthophyll are stable for as long as 150 days when stored in a refrigerator, for practically no oxidation takes place; by comparison both pigments are more stable in the first solution. It is thus possible when determining carotin in green leaves to preserve the petroleum ether solution in the refrigerator for several days without decomposition. Ether solutions of carotin and xanthophyll are unstable. At 10-20° C. the former is *more* stable than the latter while in the sunlight the ether solution of carotin is *less* stable than the corresponding solution of xanthophyll; that is, in general, carotin is more stable in the dark and xanthophyll in the light.

Color tests.—(*a*) Xanthophyll. To either an 85 per cent ethyl alcohol or a 92 per cent methyl alcohol solution of xanthophyll add concentrated hydrochloric acid and allow the color changes to take place. There first appears a brilliant green color which changes gradually to a peacock-blue, then to purple, and finally the solution becomes colorless. If at the blue stage ammonia is added to the solution, the original yellow color is restored though it is less intense. The blue color will reappear if the solution is again acidified. The alternation from blue to yellow and *vice versa* may be repeated a number of times. This reaction is characteristic of xanthophyll β.

Evaporate an alcoholic solution of xanthophyll to dryness on a steam bath; treat the residue with concentrated sulfuric acid. A deep blue color results.

Expose an alcoholic solution of xanthophyll to direct sunlight. How quickly does the solution decolorize?

(*b*) Carotin. Evaporate a petroleum ether solution of carotin to dryness on a steam bath; treat the residue with a few milligrams of ferric chloride. A green color results. Both carotin and the xanthophylls reduce ferric chloride.

Bleach a petroleum ether solution of carotin with nitrous acid. Generate the nitrous acid in the Van Slyke amino apparatus and bubble the gas from the large graduated gas buret into the solution.

*Palmer, L. S. Carotinoids and related pigments: the chromolipoids. pp. 218–247. Chemical Catalog Company, New York, 1922.

*Schertz, F. M. Some physical and chemical properties of carotin and the preparation of the pure pigment. *J. Agr. Research.*, 30, 469–474 (1925).

*Schertz, F. M. Some physical and chemical properties of xanthophyll and the preparation of the pure pigment. *J. Agr. Research*, 30, 575–585 (1925).

Expt. 224. Isolation of Carotin and Xanthophylls from Carrots.—The carrot (*Daucus carota*) is the most easily available source of the pigment, carotin; it also contains a small quantity of the xanthophyll pigments. Grate fine or scrape a well-colored carrot and boil in distilled water for one hour; this process disintegrates the cells so that the pigment may be more easily extracted. Strain through two or three thicknesses of cheesecloth and squeeze out the water from the pulp. Dry on a steam bath and pulverize. Extract the powdered carrot with ether in a Soxhlet extraction apparatus until the ether siphons over, uncolored. Concentrate the ether extract to a few cubic centimeters in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136) at room temperature; and transfer it to a separatory funnel using 80 cc. of petroleum ether.

The separation of carotin from the xanthophylls and their quantitative determination may be carried out according to the method described in Experiment 225.

Expt. 225. Quantitative Determination of Carotin and Xanthophyll.—*Separation of carotin from the Xanthophylls.*—The separation of the carotinoids depends upon the fact that methyl alcohol dissolves the xanthophylls while petroleum ether retains the carotin (Expt. 220). Remove the xanthophylls by extracting the petroleum ether solution successively with 100 cc. of 85 per cent methyl alcohol, 100 cc. of 90 per cent methyl alcohol, and twice with 50 cc. of 92 per cent methyl alcohol. If the last extraction is not colorless, extract again with 92 per cent methyl alcohol. Transfer the pigment to ether by mixing the combined methyl alcoholic extracts with 100–130 cc. of ether; slowly add distilled water; allow to separate, and then draw off the aqueous methyl alcohol layer. To overcome the difficulty sometimes encountered of transferring the pigment in the methyl alcohol to the ether, Schertz adds a saturated aqueous sodium chloride solution. Wash the ether solution of xanthophyll twice with distilled water, filter through a dry filter paper into a 100 cc. volumetric flask, clearing the solution with a few drops of absolute ethyl alcohol, and make to volume with ether.

In a similar manner wash, filter, and clear the petroleum ether solution of carotin, and make to volume with petroleum ether.

According to Schertz the petroleum ether solution of carotin may be kept in the refrigerator for several days without deterioration before colorimetric determinations are made. On the other hand determinations with the ether solution of xanthophyll must be made at once, since the latter deteriorates very rapidly.

Colorimetric determinations.—Carotin and xanthophyll may be determined colorimetrically by using a 0.2 per cent potassium dichromate

solution as a standard. Willstätter and Stoll determined the color value of this solution in terms of the pure carotinoid pigment solutions by using 5×10^{-5} molar solutions of carotin and xanthophyll, equivalent to 0.0268 gm. of carotin per liter and to 0.0284 gm. of xanthophyll per liter, dissolved respectively in petroleum ether and ether. Or expressed in terms of per cent these equal respectively a 0.00268 per cent carotin solution and a 0.00284 per cent xanthophyll solution. The relationship between the 0.2 per cent potassium dichromate solution and the 5×10^{-5} molar solutions of carotin and xanthophyll is as follows:

100 mm.	carotin	solution	equals	101 mm.	$K_2Cr_2O_7$	solution
50 "	"	"	"	41 "	"	"
25 "	"	"	"	19 "	"	"
100 "	xanthophyll	"	"	72 "	"	"
50 "	"	"	"	27 "	"	"
25 "	"	"	"	14 "	"	"

Based on this data Palmer has drawn the curves shown in Chart 2, using mm. of potassium dichromate solution as ordinates and mm. of carotin or xanthophyll solutions as abscissae.

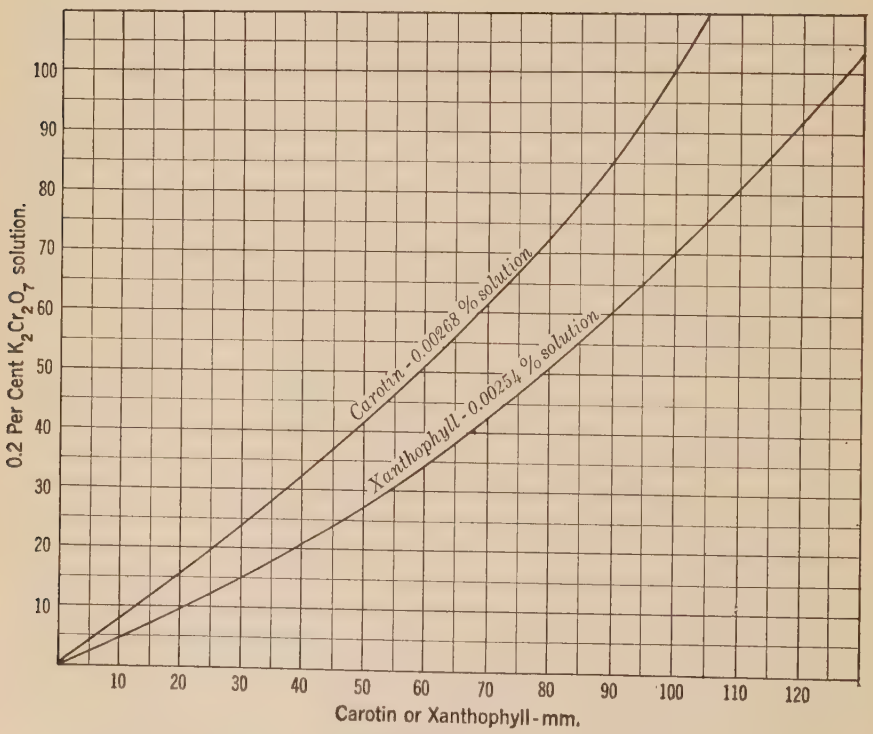

CHART 2.

By permission of L. S. Palmer, and The Chemical Catalog Company, Inc., New York.

To illustrate the method of using the curves, the example following is given: using a Duboscq colorimeter (Expt. 62), a 42.8 mm. layer of a petroleum ether solution of carotin is found to equal 10 mm. of a 0.2 per cent potassium dichromate solution. By reference to the carotin curve in Chart 2 it is seen that 10 mm. of the standard dichromate solution equals 13 mm. of 0.00268 per cent carotin solution.

That is, $x : 0.00268 :: 13 : 42.8$

$\therefore x = 0.00868$ per cent of carotin.

Tswett, M. Über das Pigment des herbstlich vergilbten Laubes. *Ber. botan. Ges.*, 26a, 94–101 (1908).

Willstätter, R. und Stoll, A. Untersuchungen über Chlorophyll. Methoden und Ergebnisse. S. 99–107. Julius Springer, Berlin, 1913.

Goerrig, Elisabeth. Vergleichende Untersuchungen über den Carotin und Xanthophyllgehalt grüner and herbstlich gelber Blätter. *Beihefte Botan. Centr.*, 35, 342–394 (1917–18).

*Palmer, L. S. Carotinoids and related pigments: the chromolipoids. pp. 259–260. Chemical Catalog Company, New York, 1922.

Schertz, F. M. The quantitative determination of carotin by means of the spectrophotometer and the colorimeter. *J. Agr. Research*, 26, 383–400 (1923). The author has used standard Lovibond slides for the colorimetric determination of carotin.

Schertz, F. M. The quantitative determination of xanthophyll by means of the spectrophotometer and the colorimeter. *J. Agr. Research*, 30, 253–261 (1925).

Schertz, F. M. Some physical and chemical properties of carotin and the preparation of the pure pigment. *J. Agr. Research*, 30, 469–474 (1925).

Schertz, F. M. Some physical and chemical properties of xanthophyll and the preparation of the pure pigment. *J. Agr. Research*, 30, 575–585 (1925).

Expt. 226. Spectroscopic Examination.—A small spectroscope, a glass vessel with parallel sides about 1 cm. apart and a strong source of light are the necessary equipment for the examination of the various plant pigments. A suitable source of light is either strong sunlight or a burner adjusted to give a luminous flame. The pigments in question, chlorophyll, the chlorophyll derivatives, carotin and xanthophyll, exhibit characteristic absorption bands in various wave lengths throughout their visible spectrum; this property may often be employed advantageously in the identification and qualitative estimation of the purity of pigment solutions. The position and intensity of the absorption bands depend somewhat on the concentration of the solution, the thickness of the layer of solution through which the light passes and particularly on the refractive index of the solvent.

(*a*) *Absorption spectrum of chlorophyll.*—Dilute a portion of the acetone extract of the leaf pigments (Expt. 216) with 5 times its volume of 85 per cent acetone, place in the observation cell, and examine with a calibrated spectroscope. Note the position and intensity of the absorption bands. The extract containing both chlorophylls *a* and *b*

exhibits 5 absorption bands in its visible spectrum. The first and most intense band is in the red which centers approximately on the *C* line and includes wave lengths 685–635$\mu\mu$; the second is a more narrow, less intense band in the wave lengths 625–610$\mu\mu$. The third and fourth bands are indistinct and narrow and are located in the 590–575$\mu\mu$ and 550–525$\mu\mu$ wave lengths respectively. The fifth band or the end absorption begins at 510$\mu\mu$ and continues toward the violet throughout the remainder of the visible spectrum.

(*b*) *Phaeophytin.*—Add a drop or two of concentrated hydrochloric acid to the acetone extract, stir, and again note the number, position and relative intensity of the absorption bands. Note that the second and third lines have shifted toward the violet and the fourth appears now as an intense band just before line, *E*, in the wave lengths 540–525$\mu\mu$.

(*c*) *Copper compound with phaeophytin.*—Examine a solution of the copper-phaeophytin compound (Expt. 221) and note that the intense absorption in the green has disappeared and that the spectrum again resembles that of chlorophyll.

(*d*) *Carotin.*—Solutions of carotin and xanthophyll in ether, alcohol and petroleum ether exhibit identical absorption spectra because of the small differences in indices of refraction between these solvents. In solutions of carotin in carbon disulfide, however, the bands are all shifted toward the red. Carotin normally exhibits two and under favorable conditions three rather poorly defined bands in the green and blue parts of the spectrum. Examine the ether solution of carotin (Expt. 224). The first band lies in the region, 492–476$\mu\mu$ and the second 459–445$\mu\mu$ wave lengths. A distinguishing feature of the carotin spectrum is that in ether, petroleum ether or alcoholic solutions, the solar line, *F*, bisects the first absorption band.

(*e*) *Xanthophyll.*—Observe the absorption spectrum of a xanthophyll solution (Expt. 229). The spectrum closely resembles that of carotin, the first band including the wave lengths 488–471$\mu\mu$ and the second 454–440$\mu\mu$.

*Willstätter, R., und Stoll, A. Untersuchungen über Chlorophyll. Methoden und Ergebnisse. 424 p. Julius Springer, Berlin, 1913.

Wood, R. W. Physical Optics. 705 p. Cf. 439–455. Macmillan Company, New York, 1923.

Baly, E. C. C. Spectroscopy. Vol. I, 2rd ed., 298 p. Longmans, Green & Company, London, 1924.

Expt. 227. Detection of Carotin in Butter Fat.—Both carotin and the xanthophylls readily lose color on oxidation. When present in oil or melted fat, they give a green color with ferric chloride. This is due to their oxidation by the ferric salt which is in turn reduced to the green ferrous salt.

Naturally colored butter from Jersey or Guernsey cows on a diet of fresh grass is best for the experiment. To prepare the butter fat place the butter in an oven at 60° C., and allow to remain several hours until the water and curd have separated from the butter fat. Filter the clear supernatant fat through a dry filter paper either in a hot water jacketed funnel or in an oven at about 60° C. If the filtered fat is not perfectly clear, refilter. Warm 5 cc. of the butter fat in a test tube and add a few milligrams of pure ferric chloride. On shaking the ferric chloride dissolves in the fat; and the yellow color rapidly gives place to green, provided the mixture is kept warm. If too much ferric chloride is added, the green color will not appear; but the fat will remain yellow because of the excess of ferric chloride in the solution. Next shake the fat vigorously with 25 cc. of hot 95 per cent ethyl alcohol; then allow to stand. The green color is extracted by the alcohol and the fat is completely decolorized. If an excess of ferric chloride has been added, the fat will be colorless, but the alcohol layer will be yellow because of the ferric chloride. It must be remembered that there is an appreciable amount of xanthophyll in naturally colored butter fat.

*Palmer, L. S., and Thrun, W. E. The detection of natural and artificial pigments in oleomargarine and butter. *J. Ind. Eng. Chem.*, 8, 614–618 (1916).

Expt. 228. Determination of Carotin in Butter Fat.—The pigment, carotin, together with cholesterol and other substances, occurs in the unsaponifiable matter of butter, butter fat, and the body fat of dairy cattle. To isolate the pigment, it is necessary to obtain the unsaponifiable matter in the highest possible degree of purity. The alcohol used in the saponification of the fat should be completely freed from aldehydes, which would otherwise form resins with the alkali. Such resins, if present, are isolated with the carotinoids and interfere with the study of the properties of the pigments.

Place 20–30 gm. of butter fat (Expt. 227) in a 250 cc. Erlenmeyer flask; for each gram of fat, add 2 cc. of 20 per cent alcoholic potassium hydroxide solution (Expt. 187). Boil the mixture for about an hour under a reflux condenser. After saponification is complete, dissolve the resulting soap in three volumes of distilled water. Cool the solution and shake it with 200 cc. of *pure* ether in a separatory funnel. Two layers separate; draw off the soap solution, and remove the ether extract. Again extract the former twice with fresh ether. The soap solution should now be colorless, and the combined ether extracts should contain the pigment and other unsaponifiable matter (Expt. 187). Wash the ether extract many times with an excess of distilled water to remove all traces of alkaline soap. To avoid emulsions, shake carefully at first, then more vigorously in subsequent washings. When the wash water

shows no alkaline reaction towards phenolphthalein, the washing is complete. Dry the golden-yellow ether solution by shaking occasionally during several hours with either neutral fused calcium chloride, or anhydrous sodium sulfate. The crude pigment is very unstable and oxidizes easily in the presence of water, especially if heated. It is necessary, therefore, to free the ether solution of the pigment from water before evaporation. Decant or filter from the drying agent and evaporate the ether in a 1000 cc. Claisen distilling flask under diminished pressure (p. 136), at room temperature. The soft residue consists of the pigment mixed with large amounts of cholesterol and traces of other unsaponifiable matter. The cholesterol may be removed by the digitonin method of Windaus: dissolve the residue in boiling 95 per cent ethyl alcohol, and precipitate the cholesterol by the addition of an excess of a hot 1 per cent solution of digitonin in 90 per cent ethyl alcohol; allow to stand over night and filter. However, the physical and chemical properties of carotin may be studied without this procedure. If the cholesterol is removed, the alcohol must be evaporated under diminished pressure. Using petroleum ether, transfer the residue to a 100 cc. calibrated volumetric flask and dilute to volume. Determine the carotin colorimetrically (Expt. 225).

Windaus, A. Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren. *Z. physiol. Chem.*, 65, 110–117 (1910).

*Palmer, L. S., and Eckles, C. H. Carotin—the principal natural yellow pigment of milk fat; its relations to plant carotin and the carotin of the body fat, corpus luteum and blood serum. I. The chemical and physiological relation of the pigments of milk fat to the carotin and xanthophylls of green plants. *J. Biol. Chem.*, 17, 191–210. Cf. 192–193 (1914).

*Palmer, L. S., and Eckles, C. H. Carotin—the principal natural yellow pigment of milk fat—Part II. Chemical and physiological relations of pigments of milk fat to carotin and xanthophylls of green plants. *Missouri Agr. Expt. Sta., Research Bull.*, 10, pp. 340–341, 1914.

Expt. 229. Determination of Xanthophyll in Egg Yolk.—The pigment, xanthophyll, together with cholesterol and other impurities occurs in the unsaponifiable matter of egg yolk. The alcohol used in the saponification should be completely freed from aldehydes, which would otherwise form resins with the alkali. Such resins, if present, are isolated with the carotinoids and interfere with the study of the properties of the pigments.

Place 100 cc. of acetone in an Erlenmeyer flask, add a well-colored egg yolk, and mix thoroughly. Heat the mixture under a reflux condenser in a water bath from 15 to 30 minutes, filter on a hot-water jacketed funnel to remove the coagulated protein, and wash with a small

quantity of hot acetone, collecting the filtrate and washings in a 250 cc. Erlenmeyer flask. Evaporate the acetone, add 50 cc. of fresh 20 per cent aldehyde- and acetone-free methyl alcoholic potassium hydroxide solution and boil the mixture for an hour under a reflux condenser. After saponification is complete, dissolve the resulting soap in three volumes of distilled water, and then extract the pigment with ether, drying and concentrating to about 50 cc. under diminished pressure according to the method given for the isolation of carotin in butter fat (Expt. 228). Transfer the ether solution of the pigment to a 100 cc. calibrated volumetric flask and dilute to volume with ether. Determine the xanthophyll colorimetrically (Expt. 225).

For a more accurate determination of xanthophyll as much cholesterol as possible should be removed. After concentrating to practical dryness, dissolve the residue in the Claisen flask in the least possible volume of hot methyl alcohol; cool in an ice mixture and filter off the cholesterol. The methyl alcohol solution contains not only the xanthophyll but traces of carotin-like pigments; to separate these, proceed according to the method given in Experiment 225. Then transfer the xanthophyll from methyl alcohol to ether.

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Palmer, L. S. Xanthophyll, the principal natural yellow pigment of the egg yolk, body fat, and blood serum of the hen. Physiological relation to the pigment of the xanthophyll of plants. *J. Biol. Chem.*, 23, 261–279 (1915).

Palmer, L. S., and Kempster, H. L. Relation of plant carotinoids to growth, fecundity, and reproduction of fowls. *J. Biol. Chem.*, 39, 299–312 (1919).

Palmer, L. S., and Kempster, H. L. The physiological relation between fecundity and the natural yellow pigmentation of certain breeds of fowls. *J. Biol. Chem.*, 39, 313–330 (1919).

Palmer, L. S., and Kempster, H. L. The influence of specific feeds and certain pigments on the color of the egg yolk and body fat of fowls. *J. Biol. Chem.*, 39, 331–337 (1919).

*Palmer, L. S. Carotinoids and related pigments: the chromolipoids. pp. 212–214. Chemical Catalog Company, New York, 1922.

II. Flavone and Flavonol Pigments

The flavones and flavonol pigments are yellow, crystalline substances, having similar properties, and high melting points. They occur most frequently as glucosides, in which form they are not as distinctly colored as in the free state. Also as glucosides they are, as a rule, readily soluble in water and alcohol, but not in ether; while as non-glucosidal pigments, they are readily soluble in alcohol, somewhat in ether, and almost insoluble in water. Shibata, Nagai, and Kishida have shown that these

yellow pigments are very widely distributed in the higher plants, not only in the flowers and leaves but also, sometimes, in the bark and wood. Wheldale found that they may be easily detected in any tissue by the intense yellow color which they give with alkalis. This reaction is best shown by the colorless parts of plants, such as white flowers.

Expt. 230. Reactions of Flavone and Flavonol pigments and Their Glucosides.—(*a*) Place a spray of flowers of the ivory-white variety of snapdragon (*Antirrhinum majus*), or any other flower, having white petals, in a glass-stoppered cylinder; add a few cubic centimeters of concentrated ammonium hydroxide; and allow to stand for a short time. A bright yellow color soon develops because of the formation of the intensely yellow salt of the flavone or flavonol pigment. Next, wash out the cylinder, add concentrated hydrochloric acid, and replace the flowers. The yellow color slowly disappears upon exposure to the hydrogen chloride gas, provided the ammonium hydroxide has not been allowed to act too long. Try sprinkling a few cubic centimeters of ammonium hydroxide on white flowers in a garden, using the same species which was tested in the laboratory. How long do they retain their yellow color?

(*b*) Prepare an extract by boiling petals of the same species of flower as that used in (*a*), with distilled water in an Erlenmeyer flask. Filter, cool, and, using a small quantity of the solution in each case, perform the following tests:

(1) Add a small amount of dilute alkali, such as sodium hydroxide. A deep yellow color develops. To this yellow solution, add a small quantity of dilute hydrochloride acid. Explain the result.

(2) Add a little dilute ferric chloride solution. A green or brown color is produced.

(3) Add a small amount of basic lead acetate solution (Expt. 141). A yellow precipitate of the lead salt is formed.

(4) To 5 cc. of the solution add about 1 cc. of concentrated hydrochloric acid and a globule of mercury the size of a pea; then add small quantities of magnesium powder from time to time. Reduction takes place with the formation of an orange-red or red to violet-red color. Add amyl alcohol and shake. Does the alcohol extract the color?

(*c*) The flavone and flavonol pigments of green leaves may be extracted with boiling water, but the dried leaves must be extracted with boiling alcohol. The alcohol also extracts the chlorophyll but usually this does not interfere with the reduction test (4), since the chlorophyll is reduced to an almost colorless product. If necessary, the chlorophyll may be removed by extraction with petroleum ether. Make an extract by boiling parsley (*Apium petroselinum* or *Petroselinum hortense*), or sumac

(*Rhus thyphina* and *Rhus glabra*) leaves with distilled water and then filter. In the same way prepare a water extract of either sumac pith, or chips of osage orange (*Maclura pomifera*). Using these extracts, perform the tests given under (*b*) and record the results.

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Expt. 231. Reduction of the Flavonol Pigments and their glucosides.—Several investigators have suggested that there is a simple chemical relationship between the flavone and flavonol pigments and the anthocyanin pigments, and that both groups may be present together in the same plant. Everest has separated the chemically related glucosides of both the flavonol pigment, myricetin, and the anthocyanidin pigment, delphinidin, from the flowers of purple-black violas. Willstätter and Mallison found that the flavonol pigment, quercetin, in an acid alcoholic solution yields, on reduction, a mixture of allocyanidin chloride and cyanidin chloride; the former being an "artificial anthocyanin," and the latter, a natural cyanidin. These non-glucosidal anthocyanidin pigments may be removed by shaking the mixture with amyl alcohol. Reduction of the flavonol glucoside, quercitrin, if carried out in the cold, produces allocyanin, the glucoside of allocyanidin, which is not soluble in amyl alcohol unless first hydrolyzed.

Using warm 95 per cent ethyl alcohol, prepare a 0.1 per cent solution of each of the following: the flavonol pigment, quercetin; and the flavonol glucoside, quercitrin. To 5 cc. of each solution, add 1 cc. of concentrated hydrochloric acid, a small globule of mercury, and from time to time very small quantities of magnesium powder. Reduction takes place very rapidly. Record the color in each case. The flavone class usually produces an orange-red color, while the flavonol class produces red to violet-red. To each of the cold quercetin, and quercitrin solutions, add amyl alcohol and shake. The alcohol extracts the color from the flavonol solution and from the solution of the glucoside, quercitrin. The quercitrin is hydrolyzed by the acid during reduction.

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Expt. 232. Quercetin, from Quercitrin.—Quercetin is one of the most important flavonol pigments. It is very widely distributed throughout the plant kingdom, occurring both in the free state and as a glucoside. In the latter form it is found most frequently combined with rhamnose, as quercitrin.

Preparation of quercetin.—Place 2.5 gm. of the flavonol glucoside, quercitrin (Expt. 174), in a 250 cc. Erlenmeyer flask, add 100 cc. of 0.6 per cent sulfuric acid, and boil under a reflux condenser on a sand bath, for an hour. When quercetin is formed in the presence of water, it shows marked imbibitional properties; hence the mixture becomes thick and at the same time changes to a color, more intensely yellow than that of the original mixture. After hydrolysis, allow the mixture to stand over night, when the quercetin becomes practically insoluble.

Filter on a small Büchner funnel, wash the residue once with cold distilled water, and preserve the filtrate for the identification of rhamnose. Remove the residue to a small beaker, mix with sufficient boiling distilled water to form a thick paste, and again filter and wash several times with small quantities of boiling water. Dissolve the quercetin in the smallest possible quantity of boiling 80 per cent ethyl alcohol and allow crystallization to take place. Filter, wash the crystals with cold distilled water, and dry at 130° C. The quercetin crystals are citron-yellow.

Identification of quercetin.—(*a*) Acetylation.—Place 0.25 gm. of quercetin in a large *dry* test tube, add 0.25 gm. of fused sodium acetate (Expt. 129) and 3 cc. of acetic anhydride. Connect the test tube with a reflux condenser and boil for an hour. Pour the reaction mixture into distilled water and allow to stand over night. Filter on a small Büchner funnel and recrystallize the penta-acetylquercetin several times from hot 70 per cent ethyl alcohol. A felt-like mass of colorless needles results. Dry at 100° C. and determine the melting-point (Expt. 128). This should be 194–196° C. To regenerate the quercetin, dissolve the dry acetyl derivative in boiling glacial acetic acid, add a few drops of concentrated sulfuric acid, and boil for a few minutes to bring about hydrolysis. Pour the mixture into a large quantity of distilled water and place in the refrigerator for 24 hours. Filter, wash the residue with distilled water, and dry at 100° C.

(*b*) *Addition product.*—Place in a dry test tube 0.25 gm. of quercetin, dissolve it in just sufficient boiling glacial acetic acid to form a saturated solution, and add a few drops of concentrated sulfuric acid. The solution turns a more intense yellow; on cooling a crystalline addition product, $C_{15}H_{10}O_7 \cdot H_2SO_4$, separates. Filter, wash the crystals with acetic acid, and dry at 100° C. Suspend the quercetin sulfate in distilled water and allow to stand over night. It is quantitatively decomposed into quercetin and sulfuric acid.

(*c*) *General reactions.*—Prepare a solution of quercetin by dissolving 0.1 gm. in 100 cc. of 95 per cent ethyl alcohol; then, using a small quantity of the solution in each case, perform the following tests:

(1) Add a small amount of dilute alkali, such as sodium hydroxide. The yellow color becomes more intense. To this yellow solution, add a small quantity of dilute hydrochloric acid. Explain the result.

(2) Add a small quantity of dilute ferric chloride solution. A dark-green color is produced.

(3) Add a little basic lead acetate solution (Expt. 141). An orange-red precipitate of the lead salt is formed.

(4) To 5 cc. of the alcoholic solution and 1 cc. of concentrated

hydrochloric acid, add small quantities of magnesium powder, from time to time. Reduction takes place with the formation of a rose-red color. Add amyl alcohol and shake. The alcohol extracts the color.

Dyeing properties of quercetin.—In the dyeing of pure wool cloth, it is generally necessary to mordant the material first and then to apply the dye. The amounts of chemicals and dyestuffs used are expressed in terms of percentage of the weight of the material to be dyed. Thus, directions, which require 3 per cent of potassium dichromate and 4 per cent of acid potassium tartrate for the bath, mean that, for each gram of cloth mordanted, 0.03 gm. of potassium dichromate and 0.04 gm. of potassium acid tartrate must be used.

(1) To mordant 1 gm. of wool cloth with *chromium,* use a 300–400 cc. beaker and prepare in it a bath containing 40 cc. of distilled water, 3 per cent of potassium dichromate and 4 per cent of potassium acid tartrate; wet the cloth with distilled water and place it in the bath; then gradually raise the solution to a temperature just below boiling and continue this for 30 minutes, stirring occasionally; add water as necessary to replace that lost by evaporation; finally wash the cloth thoroughly. Add 0.1 gm. of quercetin to 100 cc. of distilled water and boil for 5 minutes; place in this the mordanted cloth and keep at a temperature just below boiling for 30 minutes. Wash well and dry.

(2) Mordant another piece of cloth with *aluminum* in a similar manner, using 3 per cent of aluminum sulfate and 5 per cent of potassium acid tartrate. Wash the cloth thoroughly, and dye with a fresh quercetin solution. Again wash and dry.

(3) Mordant a third piece of cloth with *tin* in a similar manner, using 4 per cent of stannous chloride and 2 per cent of oxalic acid. Wash the cloth thoroughly, and dye with a fresh quercetin solution. Again wash and dry.

Compare and record the colors obtained with quercetin on the different mordants. The glucoside, quercitrin, may also be used to dye pure wool cloth. If this is done, compare the colors produced by the glucoside and its flavonol pigment.

Mordanting chemicals.—For each mordant, dissolve in sufficient distilled water to make a total volume of 100 cc. 1 gm. of one of the following substances: potassium dichromate, potassium acid tartrate, aluminum sulfate, stannous chloride, or oxalic acid.

Identification of rhamnose.—Using precipitated barium carbonate, neutralize the original filtrate to Congo red paper at boiling temperature. Filter off the barium sulfate, decolorize the filtrate with the vegetable decolorizing carbon, Norit, and again filter. Concentrate the filtrate on the water bath practically to dryness. Test a portion of the

residue for pentoses, using Bial's solution (Expt. 123*b*). From another portion, prepare the osazone (Expt. 128). Determine the melting-point of the recrystallized osazone.

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III. ANTHOCYANIN PIGMENTS

The anthocyanin pigments produce the numerous shades of blue, purple, and red which appear in the flowers, fruits, leaves, and stems of plants. They occur in the cell sap in the form of glucosides; and like the flavone and flavonol pigments, are readily soluble in water and usually in ethyl alcohol; but are insoluble in ether and chloroform. Upon hydrolysis with dilute acids the glucosides yield non-glucosidal substances, which Willstätter and Everest term anthocyanidins. For

example, pelargonin, the anthocyanin pigment from the scarlet geranium, upon hydrolysis yields one molecule of pelargonidin and two molecules of glucose; likewise, cyanin, the anthocyanin pigment from the corn flower, yields one molecule of cyanidin and two molecules of glucose. Both anthocyanins and anthocyanidins yield crystalline derivatives with acids. The authors just mentioned find that anthocyanins may be distinguished from the anthocyanidins when in aqueous acid solution, preferably sulfuric, by shaking with amyl alcohol. The amyl alcohol takes up the anthocyanidins, while the anthocyanins remain in the aqueous acid solution. Many anthocyanin pigments are unstable in neutral alcohol or aqueous solution, and readily lose color; this is caused by the conversion of the pigment into a colorless isomer. The addition of acids or certain neutral salts, such as sodium chloride and sodium nitrate, prevents or lessens this isomerism. This is due to the fact that anthocyanin is an oxonium compound having a quinonoid structure and containing tetravalent oxygen; protective addition compounds are formed with the acids or neutral salts, and these resulting oxonium salts stabilize the quinonoid structure.

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Expt. 233. Reactions of Anthocyanins and Anthocyanidins.—Extract the petals of several flowers of either the magenta snapdragon (*Antirrhinum majus*) or the crimson peony (*Paeonia officinalis*) in an Erlenmeyer flask with boiling 95 per cent ethyl alcohol under a reflux condenser, until the greater part of the pigment is removed. During the process of extraction, the anthocyanin color in the extract may disappear. What anthocyanins are present in the magenta snapdragon and peony? Filter off the alcoholic extract and use portions for the following tests:

(*a*) Add a small quantity of dilute acid and note the bright red color, which is due to the oxonium salt of the anthocyanin.

(*b*) Add a small amount of dilute sodium hydroxide solution. A green color develops.

Evaporate the remainder of the alcoholic extract practically to dryness and observe the return of the anthocyanin color. Dissolve the

residue in distilled water and make further tests, using portions of this solution in each case:

(*c*) Add ether and shake. Is the anthocyanin pigment soluble in ether?

(*d*) Add a small quantity of dilute acid. The red color is intensified.

(*e*) Add a small amount of dilute sodium hydroxide. A bluish-green or green color appears; this may finally turn yellow.

(*f*) Add a small quantity of either a basic lead acetate (Expt. 141) or a normal lead acetate solution. A bluish-green or green precipitate of the lead salt is formed.

(*g*) Add first a little dilute sulfuric acid, then amyl alcohol, and shake; the red color is not extracted by the amyl alcohol, which indicates that the pigment is an anthocyanin.

(*h*) Add a small quantity of dilute sulfuric acid and heat in a boiling water bath for half an hour. Cool, add amyl alcohol, and shake. The color passes into the amyl alcohol, which indicates that the glucosidal pigment has been hydrolyzed, forming the non-glucosidal anthocyanidin.

(*i*) Add a little hydrochloric acid and a small quantity of zinc dust. Reduction takes place and the color rapidly disappears. Filter off the solution and observe that, upon exposure to air, the color quickly returns, if the reducing action has not been too violent.

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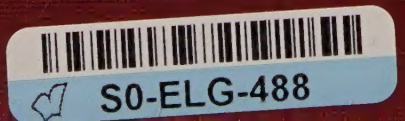
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